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1 **Antimalarial activity of human group IIA secreted phospholipase A2 in relation with**
2 **enzymatic hydrolysis of oxidized lipoproteins**

3

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13

14 **Running title: antimalarial activity of hGIIA sPLA₂**

15

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21

22 **ABSTRACT (250 words)**

23 The human group IIA secreted phospholipase A₂ (hGIIA sPLA₂) is increased in the
24 plasma of malaria patients but its role is unknown. In parasite culture with normal plasma,
25 hGIIA is inactive against *Plasmodium falciparum*, contrasting with hGIIF, hGV and hGX sPLA₂s
26 that readily hydrolyze plasma lipoproteins, release non-esterified fatty acids (NEFAs) and
27 inhibit parasite growth. Here, we revisited the anti-*Plasmodium* activity of hGIIA in
28 conditions closer to malaria physiopathology where lipoproteins are oxidized. In parasite
29 culture containing oxidized lipoproteins, hGIIA sPLA₂ was inhibitory with an IC₅₀ value of
30 150.0 ± 40.8 nM, in accordance with its capacity to release NEFAs from oxidized particles.
31 With oxidized lipoproteins, hGIIF, hGV and hGX sPLA₂s were also more potent, by 4.6-, 2.1-
32 and 1.9-fold, respectively. Using specific immunoassays, we found that hGIIA sPLA₂ is
33 increased in plasma from 41 patients with malaria over healthy donors (median (IQR): 1.6
34 (0.7-3.4) nM, versus 0.0 (0.0-0.1) nM, respectively; *P* <0.0001). Other sPLA₂s were not
35 detected. Malaria but not normal plasma contain oxidized lipoproteins and were inhibitory
36 to *P. falciparum* when spiked with hGIIA sPLA₂. Injection of recombinant hGIIA to mice
37 infected with *P. chabaudi* reduced the peak of parasitaemia, and this was effective only
38 when the level of plasma peroxidation was increased during infection.

39 In conclusion, we propose that malaria-induced oxidation of lipoproteins converts
40 these latters into a preferential substrate for hGIIA sPLA₂, promoting its parasite killing effect.
41 This mechanism may contribute to host defense against *P. falciparum* in malaria where high
42 levels of hGIIA are observed.

43

44

45 **INTRODUCTION**

46 Malaria is due to protozoan parasites of the genus *Plasmodium* that are transmitted to
47 vertebrates by mosquitoes. In mammalian hosts, *Plasmodium* spends most of its lifetime in
48 red blood cells (1). In humans, the intraerythrocytic parasite is responsible for the clinical
49 symptoms associated with malaria. The vast majority of clinical cases present as non-specific
50 febrile illnesses that are relatively easily terminated (uncomplicated malaria), but a minority
51 of cases progress to severe, life-threatening disease. According to the WHO World Malaria
52 Report 2015 (<http://www.who.int/gho/malaria/en/>) there were 214 million cases of malaria
53 globally in 2015, and 438,000 malaria deaths attributed to major complications. In this
54 context, a better knowledge of the actors of malaria physiopathology remains a key entry to
55 fight the disease. The work presented here focuses on the possible antimalarial role of a
56 family of secreted phospholipases A₂ (sPLA₂) released by mammalian host cells, with special
57 emphasis on human group IIA secreted PLA₂ (hGIIA sPLA₂).

58 sPLA₂s are structurally-conserved enzymes with a small molecular mass (14-19 kDa)
59 which catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position to release free
60 fatty acids and lysophospholipids (2-6). Mammalian sPLA₂s exhibit unique tissue and cellular
61 distributions as well as different enzymatic properties (6-9), suggesting distinct physiological
62 and pathophysiological roles for each enzyme. Besides their role in the production of lipid
63 mediators such as eicosanoids and lysophospholipids, multiple evidence indicates that
64 sPLA₂s participate in innate immunity, especially in the first line of host defense against
65 bacteria and other pathogens (10-25).

66 Among sPLA₂s, human group IIA (hGIIA) sPLA₂ is also known as the inflammatory-type
67 sPLA₂. It is a strong bactericidal agent present in inflammatory fluids and in the plasma of
68 patients with sepsis (10, 11, 17, 20, 22, 26-28). In patients with malaria, hGIIA circulates at

69 abnormally high levels, an observation originally made by Vadas and colleagues in the early
70 90s' (29, 30). However, its role in malaria has remained unknown until nowadays.

71 When the studies by Vadas *et al.* were performed, only hGIB (pancreatic-type human
72 group IB sPLA₂) and hGIIA were known in humans. Since then, additional genes coding for
73 sPLA₂s have been identified in the human genome and up to 12 genes are now identified (IB,
74 IIA, IID, IIE, IIF, III, V, X, XIIA, XIIB, otoconin-95 and the pseudogene IIC) (2, 3, 7). Very recently,
75 a genome-wide association study of non-severe malaria has suggested the possible role for
76 one or more sPLA₂s (31) present in a gene cluster containing 6 sPLA₂ genes coding for hGIIA,
77 hGIID, hGIIIE, hGIIF, and hGV (32), further strengthening our interest for the study of sPLA₂s
78 in malaria.

79 In our previous studies, we demonstrated that various venom sPLA₂s (33-36) as well as
80 several human sPLA₂s including hGIIF, hGIII, hGV and hGX sPLA₂s exert potent *in vitro*
81 antimalarial activity against *Plasmodium falciparum*, the most virulent species of human
82 parasites (33-37). However, in these typical *in vitro* infection assays of red blood cells by *P.*
83 *falciparum* where normal human serum is used, hGIIA sPLA₂ was inactive (37). We depicted
84 a mechanism by which human sPLA₂s exert their killing effect against *P. falciparum* indirectly,
85 by hydrolyzing phospholipids from human native lipoproteins present in the parasite culture
86 medium and generating lipid products such as non-esterified fatty acids (NEFAs) including
87 polyunsaturated fatty acids (PUFAs) which appeared as the key lipid products toxic to the
88 parasite and responsible for the sPLA₂-dependent parasite death (37).

89 Interestingly, it has been shown that hGIIA sPLA₂ hydrolyzes more efficiently oxidized
90 lipoproteins than their native counterparts (38-42). Oxidation of lipoproteins is observed in
91 malaria (13) and in other pathological situations including atherosclerosis, inflammatory
92 syndromes and infectious diseases (43-45). Since our above *in vitro* experimental conditions

93 using native human lipoproteins were likely not reflecting the *in vivo* physiopathological
94 conditions of malaria, we sought to re-investigate whether hGIIA and the other human
95 sPLA₂s would be more effective against *Plasmodium* in the presence of oxidized lipoproteins.

96 We found that *in vitro* oxidation of human lipoproteins converts these latter into a
97 readily hydrolyzable substrate for hGIIA sPLA₂, revealing its toxic effect towards the parasite.
98 Oxidation of lipoproteins also enhances the inhibitory effects of hGIIF, hGV and hGX sPLA₂s.
99 To provide further *in vivo* relevance of these results, plasma from healthy and *P. falciparum*-
100 infected people were analyzed for sPLA₂ content, lipoprotein oxidation and capacity to
101 inhibit *P. falciparum in vitro* growth. hGIIA sPLA₂ was increased in plasma from infected
102 patients, whereas the other sPLA₂s were not detected. The level of lipoprotein oxidation was
103 higher in malaria plasma as compared to normal plasma, and only malaria plasma was able
104 to confer *in vitro* inhibitory activity of exogenously added hGIIA sPLA₂ against *P. falciparum*.
105 The *in vivo* relevance of these observations was challenged by injection of recombinant
106 hGIIA sPLA₂ to *Plasmodium*-infected mice. A significant decrease of parasitaemia was
107 observed in mice only when the sPLA₂ was injected at the late timepoint in the course of
108 infection, at the time concomitant to increase in plasma peroxidation.

109 Together, our results suggest that the combined presence of high levels of oxidized
110 lipoproteins and hGIIA sPLA₂ might synergize to help controlling parasite growth in human
111 malaria.

112

113

114 **RESULTS**

115 **Oxidative modification of lipoproteins enhances hydrolysis by hGIIA and hGIIF but**

116 **not other sPLA₂s** — We previously reported the specific activities of different human sPLA₂s
117 on native lipoproteins (Table 1 and (37)). To analyze the anti-*Plasmodium* activity of human
118 sPLA₂s in a context more relevant to malaria where lipoproteins are oxidized (13), we
119 examined the capacity of various human sPLA₂s to hydrolyze LDL and HDL particles after *in*
120 *vitro* oxidation.

121 Hydrolysis of lipoproteins was assessed by measuring the release of non-esterified
122 fatty acids (NEFAs). Seven of the 9 recombinant catalytically-active human sPLA₂s hydrolyzed
123 oxidized and native LDL and HDL particles with the same specific activities (Table 1). In
124 contrast, hGIIA, and to a lower extent hGIIF, exhibited significantly higher activity on oxidized
125 lipoproteins. Oxidation of both LDL and HDL dramatically increased the activity of hGIIA
126 sPLA₂ whereas oxidation of LDL but not HDL slightly increased the activity of hGIIF. A slight
127 fold-change was also observed for hGIB on LDL and HDL, but this change did not reach
128 significance. However, the specific activity of hGIIA on oxidized LDL and HDL remained lower
129 than that of hGIIF, hGIII, hGV and hGX sPLA₂s, which are highly active on both native and
130 oxidized lipoproteins.

131

132 **Oxidized LDL and HDL pretreated with human sPLA₂s including hGIIA are active *in***

133 ***vitro* against *P. falciparum*** — We next compared the anti-*Plasmodium* activity of oxidized
134 lipoproteins before and after hydrolysis by recombinant human sPLA₂s. Assays were carried
135 out with lipoproteins at concentrations close to the physiological ones (0.2 mg/mL of
136 phospholipids in the culture medium). Inhibitory concentrations of sPLA₂-hydrolyzed LDL and
137 HDL against *P. falciparum* growth are presented in Table 2. Without sPLA₂ pretreatment,

138 oxidized LDLs were barely inhibitory towards *Plasmodium*, whereas oxidized HDLs were not.
139 Pretreatment with 20 nM hGIIF, hGIII, hGV and hGX sPLA_{2s}, but not other sPLA_{2s}, increased
140 the toxicity of oxidized LDL and rendered oxidized HDL toxic to the parasite. However,
141 oxidized lipoproteins were not more potent than their native counterparts after hydrolysis.
142 Oxidized LDL were less potent than native LDL after hydrolysis by hGIII sPLA₂.

143 Since the circulating levels of hGIIA sPLA₂ can be very high in severe cases of malaria
144 (29, 30) while pretreatment of oxidized lipoproteins with 20 nM hGIIA was ineffective against
145 *P. falciparum*, we evaluated the anti-*Plasmodium* potency of oxidized lipoproteins when
146 treated at higher concentrations. As shown in Table 3, pretreatment with 100 nM hGIIA
147 sPLA₂ moderately increased the toxicity of oxidized LDL whereas 250 nM induced a marked
148 toxicity of both oxidized LDL and HDL and rendered native lipoproteins toxic.

149

150 **The anti-*Plasmodium* activity of human sPLA_{2s} including hGIIA is enhanced in culture**
151 **medium containing oxidized lipoproteins** — To further analyze the effects of human sPLA_{2s}
152 in conditions closer to physiology where all classes of lipoproteins are present, a total
153 lipoprotein fraction (oxidized or not) instead of purified LDL or HDL was used without
154 enzymatic pretreatment, and incubation was prolonged for 96 h to cover two parasite intra-
155 erythrocytic cycles instead of one. As expected, hGIIF, hGV and hGX sPLA_{2s} were inhibitory in
156 culture medium containing native lipoproteins (Table 4): hGIIF and hGX sPLA_{2s} exhibited IC₅₀
157 values of 19.8 and 1.5 nM, close to the IC₅₀ values previously measured in human plasma
158 (10.7 nM and 2.9 nM, respectively (37)), whereas hGV sPLA₂ was found to be more active
159 (IC₅₀ = 21.9 nM) than in previous assays with plasma (IC₅₀ = 94.2 nM, (37)). hGIIA sPLA₂ was
160 not inhibitory in normal plasma (37) nor with native lipoproteins (Table 4). Remarkably, all
161 four sPLA_{2s} had enhanced inhibitory activities in the presence of oxidized *versus* native

162 lipoproteins. hGV and hGX sPLA₂s were 2-fold more active, and hGIIF sPLA₂ was 4.6-fold
163 more active, consistent with its increased capacity to hydrolyze oxidized lipoproteins. hGIIA
164 sPLA₂ was inhibitory in the presence of oxidized lipoproteins with an IC₅₀ value of 150 nM
165 while it was completely inactive with native lipoproteins.

166

167 **Oxidized PUFAs are not responsible for enhanced anti-*Plasmodium* activity of**

168 **sPLA₂s** – To understand why sPLA₂s exhibit greater anti-*Plasmodium* activities in assays with
169 oxidized lipoproteins, we tested whether oxidized PUFAs have greater toxicity than native
170 PUFAs. As far as we know, only one study by Kumaratilake *et al.* (46) reported the anti-
171 *Plasmodium* activity of oxidized PUFAs, namely oxidized AA and DHA. The authors found that
172 these PUFAs were more active after oxidation. Assuming that oxidized phospholipids present
173 in lipoproteins can be hydrolyzed by sPLA₂s such as hGIIA and hGX to release the
174 corresponding oxidized PUFAs (39, 47), we compared the effects of native and self-oxidized
175 AA, EPA and DHA on parasite growth in dose-response assays. When added to culture
176 medium containing human plasma, these PUFAs were inhibitory to *P. falciparum* in both
177 native and oxidized states (Table 5). The IC₅₀ values for the three PUFAs in their native state
178 were in good accordance with those reported by Kumaratilake *et al.* (46). However, once
179 oxidized, they were not more efficient, exhibiting similar (AA) or even higher (EPA, DHA) IC₅₀
180 values (Table 5). These results are thus different from those by Kumaratilake *et al.* who
181 reported a \approx 1.5-fold (AA) and \approx 2.0-fold (DHA) better capacity of these PUFAs to inhibit
182 *Plasmodium* after oxidation (46). The discrepancy might come from differences in the
183 amount and molecular identity of oxidation products as well as differences between
184 experimental settings for oxidation. Anyway, this indicates that there is no clear-cut
185 difference between the capacity of oxidized *versus* native PUFAs to inhibit *Plasmodium*

186 growth, and suggests that the enhancement of the anti-*Plasmodium* activity of sPLA₂s in the
187 presence of oxidized lipoproteins might rather come from an increased activity of sPLA₂s at
188 hydrolyzing oxidized particles and from prolonged incubation during the inhibition assay,
189 resulting in a greater production of various toxic lipids, beyond the oxidized ones.

190

191 **Circulating levels of hGIIA sPLA₂, but not hGIIF, hGV or hGX sPLA₂s, are increased in**
192 **uncomplicated *P. falciparum* malaria** — To provide a pathophysiological relevance of our *in*
193 *vitro* results, we tested the sPLA₂ enzymatic activity and the presence of hGIIA, hGIIF, hGV
194 and hGX sPLA₂s in plasma from 41 *P. falciparum*-infected Vietnamese adults *versus* 28 non-
195 parasitized, healthy donors from the same geographic area. None of the parasitized
196 individuals presented signs of complicated malaria at the time of blood sampling. As shown
197 in figure 1A, sPLA₂ enzymatic activity was dramatically increased in the plasma of *P.*
198 *falciparum*-infected patients relative to healthy donors (median (interquartile range: IQR):
199 260.0 (148.5-367.1) *versus* 16.20 (10.46-29.06) cpm/min x μL respectively, *P* value < 0.0001).
200 No correlation was found between the level of enzymatic activity and blood parasitaemia (*P*
201 value = 0.8079, Spearman's *r* = 0.03969, not shown).

202 Plasma samples were next analyzed for the presence of hGIIA, hGIIF, hGV and hGX
203 sPLA₂s by a sandwich ELISA-like method (Time-resolved fluoroimmunoassay, TR-FIA) using
204 specific antibodies and assays for each sPLA₂ (27). Protein levels of hGIIA sPLA₂ were
205 significantly increased in the plasma from parasitized patients (median (IQR): 1.6 (0.7-3.4)
206 nM, with a maximum value of 9.1 nM, as compared to the plasma from healthy donors
207 (median (IQR): 0.0 (0.0-0.1) nM) (*P* value <0.0001) (figure 1B). In contrast, hGIIF, hGV and
208 hGX sPLA₂s were not detected in the parasitized plasma (not shown), indicating that these
209 enzymes do not circulate, at least at detectable levels, in uncomplicated malaria. In the

210 parasitized plasma, sPLA₂ enzymatic activity and hGIIA protein concentration were highly
211 correlated (Figure 1C; P value <0.0001, Spearman's $r = 0.9542$), indicating that hGIIA sPLA₂ is
212 the predominant sPLA₂ responsible for the circulating enzymatic activity. No correlation was
213 found between the plasma concentration of hGIIA and the parasitaemia from infected
214 donors (Figure 1D; P value = 0.6684, Spearman $r = 0.0689$).

215

216 **Malaria but not normal plasma inhibits *in vitro* parasite growth when spiked with**
217 **recombinant hGIIA sPLA₂.**

218 The capacity of hGIIA sPLA₂ endogenously present in the plasma from malaria patients
219 at inhibiting *Plasmodium* development was evaluated in conventional *in vitro* culture
220 conditions. Ten plasma samples containing various levels of endogenous hGIIA sPLA₂ up to 9
221 nM were tested (Figure 2A). It is important to note that in our culture conditions where
222 RPMI was supplemented with 8% human plasma, the final hGIIA concentrations were only in
223 the range of 0.1 to 1 nM. The growth rates of *P. falciparum* measured in the presence of
224 plasma from the different malaria patients was variable but not significantly lower than that
225 measured in the presence of plasma from different healthy donors (median growth rate
226 (IQR): 6.1 (4.3-14.4) in malaria plasma *versus* 8.1 (6.7-9.5) in normal plasma, $P = 0.4278$;
227 Figure 2A). We then evaluated the possible role of hGIIA sPLA₂ present in the diluted plasma
228 by adding to the culture medium LY311727, a specific active site inhibitor of hGIIA sPLA₂ (8).
229 Addition of LY311727 did not modify the parasite growth rate in the plasma samples
230 (multiplication factor after 96 h of cultivation: median (IQR): 7.08 (2.87-17.5) without
231 LY311727 *versus* 6.65 (4.14-10.7) with LY311727, $P = 0.1550$) (not shown). The absence of
232 effect of LY311727 i) indicates that the variable capacity of the different malaria plasma to
233 support parasite growth is not due to hGIIA, and ii) might be explained by too low

234 concentrations of endogenous hGIIA in our assay conditions to be effective at inhibiting the
235 growth of *Plasmodium*. To test this possibility, *P. falciparum* was grown for 96 h in plasma
236 from malaria patients *versus* healthy donors spiked with 100 nM recombinant hGIIA sPLA₂.
237 This concentration was chosen based on hGIIA plasma concentrations reported in severe
238 cases of malaria (29, 30). As shown in Figure 2A, hGIIA spiked in malaria but not normal
239 plasma inhibited parasite development, demonstrating that 1) the concentration of
240 endogenous hGIIA in our assay conditions was not sufficient to inhibit parasite growth but
241 that higher concentrations were clearly effective, and 2) malaria-induced modifications of
242 plasma (possibly including oxidation of lipoproteins) is required to reveal the anti-
243 *Plasmodium* activity of hGIIA. We also tested the spike of malaria plasma with 10 nM hGIIIF
244 or 0.5 nM hGX recombinant sPLA₂s for comparison. Both enzymes were inhibitory for *P.*
245 *falciparum* in malaria plasma and found more active than hGIIA sPLA₂ (Figure 2B), as
246 anticipated from the *in vitro* results with human native plasma.

247

248 **Lipoproteins from *P. falciparum*-infected plasma are oxidized** — To add further
249 support to our hypothesis that the anti-*Plasmodium* activity of hGIIA sPLA₂, and may be of
250 other sPLA₂s, is promoted by the presence of oxidized lipoproteins in the plasma from
251 infected patients, we evaluated the level of lipoproteins and their oxidative status in malaria
252 plasma *versus* controls. In accordance with previous observations reporting a drop in HDL
253 content in patients' blood during malaria (48, 49), the level of lipoproteins, as measured by
254 total PC concentration was found to be lower in plasma from malaria patients as compared
255 to healthy donors (median (IQR): 1.20 (1.00-1.46) mg/mL *versus* 1.36 (1.19-1.68) mg/mL, *P* =
256 0.0158) (Figure 3A). Oxidation of lipoproteins in plasma was estimated using the specific
257 Mercodia oxidized LDL immunoassay kit (see methods). LDL oxidation was clearly increased

258 in the plasma from malaria patients as compared to healthy donors (median (IQR): 33.13
259 (25.65-39.79) vs 15.41 (12.13-18.69) U/L, $P < 0.001$) (Figure 3B).

260 Together, our results demonstrate that malaria-induced modifications of plasma
261 include oxidation of lipoproteins that most likely promotes the anti-*Plasmodium* activity of
262 hGIIA sPLA₂ and enhances the inhibitory capacity of hGIIF and hGX sPLA₂s, when spiked in
263 malaria plasma.

264

265 **The anti-*Plasmodium* activity of hGIIA sPLA₂ in malaria plasma requires its catalytic**
266 **activity** — To demonstrate that the anti-*Plasmodium* activity of hGIIA sPLA₂ in malaria
267 plasma requires enzymatic hydrolysis of oxidized lipoproteins, we first assessed the ability of
268 the enzyme to hydrolyze phospholipids from malaria plasma. Plasma samples were
269 incubated with hGIIA sPLA₂ as well as hGX sPLA₂, since this latter actively hydrolyzes both
270 native and oxidized lipoproteins. hGIIA sPLA₂ did not release NEFAs from healthy plasma,
271 whereas small but significant release occurred from malaria plasma ($P = 0.0093$) (Figure 4A).
272 As expected, hGX sPLA₂ induced a net increase in NEFAs from both malaria ($P = 0.0020$) and
273 healthy donor ($P = 0.0019$) plasma (Figure 4A).

274 Second, we tested whether the enzymatic activity is essential for the anti-*Plasmodium*
275 effect of hGIIA sPLA₂. Parasite growth inhibition assays were performed using the H48Q
276 catalytically-inactive mutant of hGIIA (<0.5% of WT enzymatic activity (50)). The parasite
277 development was not affected in malaria plasma spiked with the H48Q mutant, indicating
278 that hydrolysis of plasma phospholipids is required for parasite inhibition (Figure 4B).

279 Last, we analyzed the relationship between *Plasmodium* inhibition in malaria plasma
280 supplemented with hGIIA sPLA₂ and the amount of NEFAs released by the enzyme in the
281 culture supernatant. We found that both parasite inhibition and NEFA amount in the culture

282 medium increase with the time of incubation and concentration of hGIIA sPLA₂ (Figure 4C).
283 Interestingly, a small but noticeable inhibition of parasite development was observed in the
284 presence of 20 nM hGIIA beyond 48 h of incubation, suggesting a possible long-term effect
285 induced by relatively low concentrations of the enzyme.

286 Together, these results indicate that the anti-*Plasmodium* activity of hGIIA sPLA₂
287 observed in the plasma from malaria patients is mediated by hydrolysis of oxidized
288 lipoproteins.

289

290 **Recombinant hGIIA sPLA₂ inhibits parasite development in *Plasmodium*-infected**
291 **mice only when injected at the time of plasma peroxidation.**

292 To challenge our hypothesis that hGIIA sPLA₂ might help controlling parasite
293 development in an *in vivo* situation where lipoprotein oxidation does occur, we injected
294 recombinant hGIIA sPLA₂ to C57BL/6J mice infected with *P. chabaudi* at different time points
295 post-inoculation. The recombinant enzyme was injected twice daily for 3 consecutive days,
296 either at the onset of the patent phase (when the first parasites are observed on blood
297 smears) or later, just prior to the parasitaemia peak. Interestingly, a significant decrease by
298 about 20% of the parasitaemia peak was observed after the late injection of hGIIA (Figure
299 5B), but not when hGIIA was injected at early time points (Figure 5A).

300 To assess the potential involvement of oxidized lipoproteins in the *in vivo* anti-
301 *Plasmodium* effect of hGIIA, the plasma from infected mice was analyzed for lipid
302 peroxidation. The levels of TBARS were increased at day-14 following parasite inoculation
303 but not before (Figure 5C), i.e. at the time coincident with the one when injection of hGIIA
304 was found to be effective against *Plasmodium* infection.

305

306 DISCUSSION

307 Since the discovery in the early 90's of an increase of hGIIA sPLA₂ in the plasma from
308 malaria patients (29, 30), no comprehensive study on the potential role of this enzyme has
309 been made in the context of malaria physiopathology. Interestingly, an association of plasma
310 PLA₂ enzymatic activity and related phospholipid products with brain swelling in pediatric
311 cerebral malaria has been reported in 2015 (51), suggesting a role of hGIIA sPLA₂ in the
312 inflammatory events operating in cerebral malaria. More recently, a possible role for the
313 gene cluster encoding multiple sPLA₂ (genes for hGIIA, hGIID, hGIIIE, hGIIF and hGV sPLA₂s)
314 has been suggested from results of a genome-wide association study performed on non-
315 complicated malaria susceptibility (31), highlighting the possible role for one or more human
316 sPLA₂s in malaria.

317 In line with the above findings, we provide here the first evidence that hGIIA sPLA₂ and
318 maybe other sPLA₂s might contribute as host defense factors to control *Plasmodium*
319 development. Our results indicate that hGIIA, as well as some other sPLA₂s, might operate
320 through hydrolysis of lipoproteins, and more precisely oxidized lipoproteins that are
321 produced during the course of *Plasmodium* infection in malaria (13). This was demonstrated
322 by combining a series of *in vitro* studies on parasite infection, analysis of plasma from
323 malaria patients and finally by *in vivo* analysis of the anti-*Plasmodium* effect of hGIIA sPLA₂ in
324 a mouse model of chronic malaria.

325 We previously demonstrated that hGIIF, hGIII, hGV and hGX but not hGIIA sPLA₂s
326 inhibit the *in vitro* growth of *P. falciparum* through hydrolysis of native lipoproteins present
327 in human plasma (37). We now show that oxidized lipoproteins are also substrates for these
328 enzymes and participate to the inhibition of parasite growth, at least *in vitro*. Focusing on
329 hGIIA sPLA₂ because of its presence in the plasma from malaria patients, we also show that i)

330 hGIIA hydrolyzes much more potently oxidized LDL and HDL than their native counterparts
331 (where no detectable hydrolysis was observed), a finding in accordance with previous results
332 (38, 39, 42), and ii) hGIIA exhibits anti-*Plasmodium* activity only in the presence of oxidized
333 lipoproteins.

334 In normal culture conditions, hGIIF, hGIII, hGV and hGX sPLA₂s hydrolyze lipoprotein
335 phospholipids, producing toxic free fatty acids, mainly long chain PUFAs (37). It is known that
336 hGIIA sPLA₂ has little activity on native lipoproteins but higher activity on oxidized
337 counterparts ((38, 39, 42) and this study). Identification and quantification of the
338 lysophospholipids and fatty acids produced upon hydrolysis of native lipoproteins by hGIIA
339 and other sPLA₂s have been reported by us and others (37-39). It is known also that hGIIA
340 sPLA₂ hydrolyzes oxidized lipoproteins to generate non-oxidized lipids and likely oxidized
341 products (38, 39, 41, 42). *In vitro* and *in vivo* antimalarial properties of native as well as
342 oxidized lipids, especially PUFAs, has been reported elsewhere (46, 52). Kumaratilake *et al.*
343 have shown that oxidized PUFAs are more active than native PUFAs against *P. falciparum in*
344 *vitro* (46). However, we did not find evidence that oxidized PUFAs are more toxic than native
345 PUFAs, yet PUFAs were indeed toxic. These differences might be due to experimental
346 variations between the two studies, and obviously, additional investigations will be
347 necessary to solve the question of the role of oxidized lipids in the anti-*Plasmodium* activities
348 of sPLA₂s. Considering the other sPLA₂s, it is interesting to note that hGIIF, hGV and hGX
349 sPLA₂s displayed increased capacities to inhibit *Plasmodium* when incubated with oxidized
350 lipoproteins from the whole lipoprotein fraction and for a long incubation time (96 h, i.e.
351 two parasite cycles), whereas LDL or HDL first purified, and then oxidized and prehydrolyzed
352 with 20 nM of those sPLA₂s did not exhibit greater toxicity than the native ones in the
353 classical 48 h inhibitory assay. This indicates that experimental conditions are crucial to

354 reveal the anti-*Plasmodium* properties of sPLA₂s, and suggests that a specific
355 pathophysiological environment is a key factor in promoting their enzymatic and biological
356 activities. In line with our findings, many studies have now shown that hGIIA sPLA₂ is only
357 active when acting on specific "non-cellular" and cellular phospholipid substrates, including
358 microparticles, exosomes or free mitochondria released by activated human platelets or
359 other immune cells, or damaged and apoptotic cells after oxidation or scramblase-mediated
360 phosphatidylserine externalization (5, 53-64).

361 We measured concentrations of hGIIA sPLA₂ up to 9 nM in the plasma from a
362 Vietnamese cohort of 41 patients with uncomplicated malaria. These concentrations are
363 consistent with a previous study by Vadas *et al* in a small cohort of 14 Canadian adult
364 patients with uncomplicated malaria (29), where the mean level of hGIIA sPLA₂ was $12.1 \pm$
365 4.8 nM (169.7 ± 67.3 ng/mL). We found that *P. falciparum* develops normally in such malaria
366 plasma (diluted to 8%, i.e. at a relatively low concentration of hGIIA in the nM range)
367 although it contains oxidized LDL. In a subsequent study by Vadas *et al* on Malawian children
368 with severe malaria, the mean level of hGIIA sPLA₂ was estimated to be around 70 nM, with
369 the most severe cases exhibiting levels up to 200 nM (30). Of note, in these studies including
370 ours, plasma were likely collected at different times during the course of infection, and it is
371 not known how varies the concentration of hGIIA during the infection and according to levels
372 of parasitaemia. In line with such high concentrations, we show that addition of 100 nM
373 hGIIA sPLA₂ to uncomplicated malaria plasma induces significant parasite inhibition, whereas
374 the same addition of hGIIA to normal plasma is ineffective. Furthermore, it should be
375 considered that hGIIA sPLA₂ has high affinity for various heparan sulfate proteoglycans lining
376 the vascular endothelium and various cells, which likely acts as a reservoir of hGIIA in the
377 circulation, and may lead to an underestimation of the actual concentration of hGIIA in

378 blood (65-68). This is supported by the study by Nakamura et al. where the hGIIA sPLA₂
379 activity measured in plasma was increased by about 3-fold between heparinized and non-
380 heparinized patients (69). This indicates that conventional collection of plasma or serum
381 without heparin pretreatment leaves behind numbers of hGIIA molecules bound to the
382 heparan sulfates in the vasculature or circulating immune cells. With these data in mind, the
383 100 nM concentration of hGIIA sPLA₂ used in our *in vitro* experiments may not be that far
384 from the concentration in the vascular compartment of malaria patients, especially in severe
385 cases of malaria. A last point to consider when comparing our *in vitro* data with what might
386 occur *in vivo* is the fact that hGIIA needs an appropriate substrate to be effective, in our case
387 oxidized lipoproteins. Besides the various levels of hGIIA circulating in patients, the quality
388 and quantity of this particular substrate might also vary among individuals, leading to
389 variable anti-*Plasmodium* host response.

390 By investigating the effects of injection of recombinant hGIIA sPLA₂ to parasitized mice,
391 we show that *Plasmodium* development is reduced in those mice. Interestingly, the enzyme
392 is effective only when injected in the late course of infection (at days 12-14 p.i.) and not at
393 earlier time points (at days 8-10 p.i.). Consistent with the *in vitro* results, plasma
394 peroxidation was increased at day 14 but not at day 8, in line with the time window at which
395 the injected hGIIA sPLA₂ is effective against *Plasmodium* in parasitized mice.

396 It must be noticed that the drop in parasitaemia induced by injection of hGIIA is rather
397 modest, i.e. about 20%. This may reflect a transient effect of the injected enzyme due to its
398 rapid *in vivo* clearance or capture by the vascular wall components, and hence lower
399 bioavailability of the soluble enzyme at hydrolyzing plasma oxidized lipoproteins. This
400 situation may differ from that in infected patients where the enzyme is continuously
401 produced by the sustained inflammation and circulates in plasma at steady state high levels.

402 The use of transgenic mice continuously overexpressing the human enzyme may be more
403 relevant in this context, as they were previously used to demonstrate the antibacterial effect
404 of hGIIA sPLA₂ *in vivo* (16-18, 70-72) and have been shown to have altered lipoprotein
405 profiles (73, 74).

406 One may also consider that the *in vivo* antimalarial activity of hGIIA sPLA₂ results from
407 multiple mechanisms of action, with the release of PUFAs directly toxic to the parasite being
408 only one among several mechanisms. Indeed, there is considerable evidence that hGIIA
409 sPLA₂ is an important effector of the innate immune response and may activate platelets or
410 neutrophils among different immune cells involved in malaria (22, 62, 63, 75, 76). The
411 production of lipid mediators by sPLA₂s, as an integral component of the inflammatory
412 reaction, plays a major role in protecting the host against invading pathogens (3, 5, 22).
413 However, lipid products might also induce deleterious effects to the host, as exemplified by
414 recent metabolomics analyses showing a positive association between lipid products of the
415 PLA₂ pathway and brain swelling in pediatric cerebral malaria (51).

416 Concerning the possible *in vivo* role of other human sPLA₂s, we showed that
417 exogenous hGIIF and hGX sPLA₂s are active against *Plasmodium* in plasma from malaria
418 patients as well as healthy donors, in accordance with their ability to hydrolyze lipoproteins
419 under native or oxidized states. These sPLA₂s were however not detected in the plasma of *P.*
420 *falciparum*-infected or healthy subjects. It is tempting to speculate that they may act locally
421 at sites relevant to *Plasmodium* infection. Indeed, while there is now considerable evidence
422 that hGIIA sPLA₂ is the only sPLA₂ highly present in the circulation under pathological
423 conditions associated with inflammation, tissue injury or infection, there is little evidence for
424 the presence of other sPLA₂ isoforms in serum, except for hGIB sPLA₂ in some disease
425 situations (27, 77). However, several sPLA₂s other than hGIIA are also involved in various

426 types of infection, suggesting that these sPLA₂s exert their effects in diverse pathologies
427 locally, within the microenvironment of the disease, and either in an autocrine or paracrine
428 manner (3, 5, 6). Examples include studies on the role of GIII, GV and GX sPLA₂s after
429 infection with various pathogens (19, 21, 78-81), GIII and GX sPLA₂s in cancer (6, 82, 83), or
430 GIIF, GIII, GV and GX sPLA₂s in cardiovascular or skin diseases, and allergy or asthma (84-89).
431 It is also interesting to note that hGX sPLA₂ has been found in atherosclerotic plaques but
432 not in plasma from patients with atherosclerosis (90), suggesting that the enzyme is
433 produced locally but not systemically. All together, it is possible that sPLA₂s other than hGIIA
434 also contribute to malaria within the vascular wall or *via* local secretion from immune cells,
435 even though these enzymes are not detected in plasma.

436 In summary, we have shown that the concomitant presence of high concentrations of
437 hGIIA sPLA₂ and oxidation of lipoproteins, as found in the serum of malaria patients, might
438 participate to the host defense mechanism against *P. falciparum*. The mechanism of action
439 would be based on a synergistic effect between the enzyme and its capacity to hydrolyze
440 oxidized lipoproteins serving as one of its preferred substrates to release toxic lipids for
441 *Plasmodium*, yet other indirect mechanisms may occur. By extrapolation, this mechanism of
442 action of hGIIA on oxidized lipoproteins might also be involved in other pathological
443 conditions where both hGIIA sPLA₂ and oxidized lipoproteins are present, including
444 atherosclerosis or other infectious diseases. It also remains to determine if other sPLA₂s such
445 as hGIIF, hGIII, hGV and hGX which are active *in vitro*, may also be active in an *in vivo*
446 situation, even though these enzymes are not found in plasma from malaria patients.

447 MATERIALS AND METHODS

448 **Materials** — The FcB1 strain of the human *Plasmodium: P. falciparum* (Columbia) and the
449 864VD strain of the murine *Plasmodium: P. chabaudi chabaudi* used in this work was from
450 the Unicellular Eukaryotes Collection from the National Museum of Natural History (MNHN-
451 CEU-224-PfFcB1). The 864VD strain of the murine *Plasmodium P. chabaudi chabaudi* was
452 from the MCAM Research Unit's *Plasmodium* collection. C57BL/6JOlaHsd mice for the *in vivo*
453 assays were from Envigo RMS SARL (Gannat, 03800 France). Plasma and red blood cells for
454 the *in vitro* studies were from the O– or A+ blood groups and were supplied by the French
455 National Agency for Blood (Etablissement Français du Sang (EFS)), convention reference: C
456 CPSL UNT 13/EFS/126. RPMI 1640 and Albumax II® were from Life Technologies (Cergy
457 Pontoise, France). Diff-Quick staining reagents were from Medion Diagnostics AG.

458 The NEFA-C and the Phospholipid B kits, respectively used for quantitative
459 determination of non-esterified fatty acids (NEFAs) and phospholipids, were from WAKO
460 Chemicals (Oxoid S.A., Dardilly, France). The oxidized LDL ELISA kit was from Mercodia SAS
461 sales in France. Purified recombinant human sPLA_{2s} and the hGIII sPLA₂ domain were
462 prepared as described (91). The sPLA₂ inhibitor LY311727 (3-[[3-(2-Amino-2-oxoethyl)-2-
463 ethyl-1-(phenylmethyl)-1*H*-indol-5-yl]oxy]propyl]-phosphonic acid (164083-84-5)) that
464 targets hGIIA sPLA₂ was from Sigma.

465 The polyunsaturated fatty acids (PUFAs): arachidonic acid (AA, C20:4, n-6),
466 eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3) were
467 from Cayman Chemicals (Interchim). ³H-hypoxanthine monohydrochloride (370 GBq·1.11
468 TBq/mmol) was from Perkin-Elmer. Other high quality grade biochemical reagents were
469 from Sigma.

470

471 **Methods**

472 ***P. falciparum* cultivation** — In routine culture conditions, *P. falciparum* was grown in
473 red blood cells from the A+ group at 2% haematocrit and 2-4% parasitaemia, in RPMI
474 supplemented with 11 mM glucose, 27.5 mM NaHCO₃, 100 UI/mL penicillin, 100 µg/mL
475 streptomycin, adjusted to pH 7.4 (basic medium), supplemented with 8% heat-inactivated
476 human A+ plasma (complete medium), according to the procedure of Trager and Jensen (92).
477 When specified, the serum substitute Albumax II® (0.5% w/v final) was used in culture
478 medium instead of heat-inactivated human plasma. Culture flasks were gassed with 91% N₂,
479 6% O₂ and 3% CO₂ before being incubated at 37°C. In those conditions, the intra-erythrocytic
480 cycle of the FcB1 strain was 48-h long. Parasitaemia (%) was established by optical
481 examination of Diff-Quik-stained smears from the formula = [infected erythrocytes number /
482 total erythrocytes number] x 100.

483

484 **Purification of lipoproteins and oxidation** — Non-fasted human plasma was split into
485 aliquots and frozen at -20°C. One aliquot was thawed to prepare LDL and HDL by differential
486 centrifugation, according to Havel et al. (93). Briefly, chylomicrons and VLDL were removed
487 by a first round of centrifugation at 1.006 g/mL density, then LDL and HDL were purified
488 separately by successive centrifugations at 1.053 g/mL and 1.210 g/mL densities,
489 respectively. When specified, total lipoprotein fraction was purified by a single run of plasma
490 at 1.210 g/mL density. Lipoproteins were dialyzed at 4°C against NaCl 9 g/L, then against
491 RPMI, and sterilized by 0.2 µm filtration. Oxidation was achieved by storing lipoproteins in a
492 transparent flask at room temperature under sterile air exchange for 14 days. Native
493 lipoproteins were prepared from another aliquot of the plasma just prior to assays.
494 Experiments were performed within 1 week of lipoprotein storage at 4°C under N₂ in the

495 dark. Phosphatidylcholine (PC) content of lipoproteins was measured by using the
496 Phospholipid B dosage kit, according to the manufacturer's instructions.

497

498 **Oxidation of PUFAs** — Concentrated solutions of commercial AA (76.5 mM), EPA (60
499 mM) and DHA (100 mM) in ethanol were stored frozen under N₂. An aliquot of 20 µL of each
500 PUFA was allowed to dry in a glass tube and then exposed to air and light for 1 week under
501 sterile conditions. Dried PUFAs were re-suspended into 100 µL CHCl₃, and then diluted 1/20
502 in RPMI containing 8% heat-inactivated human plasma.

503 Prior to dose-response assays, non-oxidized PUFAs were prepared from another 20 µL
504 aliquot of each concentrated solution, mixed to 80 µL of CHCl₃ and diluted into culture
505 medium as above.

506

507 **Measurement of lipid oxidation in lipoproteins and plasma** — The level of TBARs
508 (thiobarbituric acid reactive substances) was determined as a marker of lipid peroxidation in
509 purified human lipoproteins (94, 95). Briefly, 150 µL of diluted sample containing 0.2%
510 butylated hydroxytoluene was mixed with an equal volume of 0.25 N HCl containing 15%
511 trichloroacetic acid and 0.4% thiobarbituric acid. The mixture was heated at 85°C for 15
512 minutes and then cooled on ice. 150 µL of butanol was added, the sample was vortexed, and
513 then let stand on ice for phase separation to occur. The butanol phase was taken for TBARs
514 determination at 515 nm excitation and 550 nm emission wavelengths on a
515 spectrofluorometer (AMINCO-Bowman series 2). 1,1,3,3-tetraethoxypropane was used as an
516 external standard. Three independent measurements with plasma lipoproteins from
517 different donors were performed. Before oxidation, TBARS ranged between 0.30-0.50 and
518 0.05-0.15 nmoles of malondialdehyde (MDA)/mg of protein in LDL and HDL, respectively.

519 After two weeks of air-light oxidation, TBARS concentration had raised to 1.50-2.50 (LDL) and
520 0.30-0.50 (HDL) nmoles of MDA/mg of protein. For comparison, TBARS in LDL and HDL
521 purified from the serum of malaria cases were reported to be around 1.0 and 0.2 nmol/mg
522 of protein, respectively (13).

523 Due to the presence in plasma of substances other than MDA and susceptible to react
524 with thiobarbituric acid (96), the level of lipoprotein oxidation in crude human plasma was
525 determined by quantifying oxidized LDL using the Ox-LDL ELISA kit from Mercodia, a
526 sandwich ELISA based on the mouse monoclonal antibody 4E6, which is directed against a
527 conformational epitope in oxidized ApoB-100. The ELISA assay was performed according to
528 the manufacturer's instructions. Oxidation of mouse plasma was measured by quantifying
529 TBARS because the Mercodia kit is not appropriate for mouse plasma.

530

531 **Hydrolysis of lipoproteins and plasma by sPLA₂** — Native and oxidized LDL and HDL
532 purified from the same batch of human plasma were adjusted to 1 mg phospholipid/mL in
533 RPMI supplemented with 1 mM CaCl₂. They were incubated with and without recombinant
534 sPLA₂ for various times at 37°C. sPLA₂s were used at different concentrations. hGIB was used
535 at 100 nM (number of independent experiments, n = 4), hGIIA was used at 50, 100 and 250
536 nM (n = 7), hGIID was used at 100 and 200 nM (n = 2), hGIIE was used at 200 and 250 nM (n
537 = 2), hGIIF was used at 30 and 40 nM (n = 6), hGIII was used at 50 and 100 nM (n = 4), hGV
538 was used at 10, 40 and 50 nM (n = 6), hGX was used at 5, 10 and 20 nM (n = 7), hGXIIA was
539 used at 100 and 200 nM (n = 2). NEFAs were measured at different time points using the
540 NEFA-C kit (WAKO) following manufacturer's instructions. Values were normalized by
541 subtracting NEFAs measured from lipoproteins incubated without sPLA₂. Specific activity was
542 deduced from the linear part of the curve [NEFA] = f (t).

543 To assess the ability of hGIIA and hGX sPLA₂s to hydrolyze phospholipids in plasma
544 from *P. falciparum*-infected patients versus healthy donors, 1 mM final CaCl₂ was added to
545 each plasma sample and then enzymes were added at 400 nM (hGIIA) and 8 nM (hGX) final
546 concentration. Samples were incubated overnight at 37°C. Control for endogenous release of
547 NEFAs was without added sPLA₂. NEFA content before and after incubation was determined
548 using the NEFA-C kit, according to manufacturer's instructions.

549

550 **Anti-Plasmodium activity assays with sPLA₂-hydrolyzed lipoproteins** — The ability of
551 human sPLA₂s to promote the toxicity of lipoproteins was tested in dose-response assays as
552 described (37). Briefly, LDL and HDL (0.6 mg phospholipid/mL in RPMI) were incubated
553 overnight at 37°C with 20 nM sPLA₂ or alone, then tested for parasite inhibition at a LDL and
554 HDL final concentration of 0.2 mg/mL (ie close to physiological concentrations) in RPMI 0.5%
555 Albumax II® for 48h in normal culture conditions. Albumax II® instead of human plasma was
556 used throughout the test to avoid any contribution of lipoproteins from human plasma.
557 Incubations with each sPLA₂ added alone were also performed to check for lipoprotein-
558 independent toxicity of sPLA₂s in these conditions. Assays analyzing the anti-*Plasmodium*
559 effect of lipoproteins pre-treated with higher concentrations of hGIIA sPLA₂ (100 nM and
560 250 nM) were performed similarly. Assays analyzing the anti-*Plasmodium* effect of sPLA₂s co-
561 incubated with total lipoprotein fraction but without pretreatment were carried out on two
562 parasite cycles (i.e. 96 h).

563

564 **Anti-Plasmodium activity assays with oxidized PUFAs** – Dose-response assays with
565 oxidized and non-oxidized AA, EPA and DHA were performed as described in (37) with some
566 modifications. Decreasing concentrations of each PUFA were established by 3-fold dilution

567 steps in culture medium, distributed in a 96-well plate (50 μ L/well) and mixed with a culture
568 of *P. falciparum* (0.25% parasitaemia, 4% haematocrit, 50 μ L/well). Parasites were allowed to
569 grow for 48 h in normal culture conditions, then 9.25 kBq 3 H-hypoxanthine were added per
570 well. After an additional 48 h incubation period, plates were frozen and the experiment
571 proceeded as in (37).

572

573 **Human plasma collection in Vietnam** — Venous whole blood samples were collected
574 in Acid Citrate Dextrose (ACD) (BD, India) vacutainers from patients attending commune
575 health centers in the Binh Phuoc province, VietNam. Malaria positivity was evaluated by
576 using the rapid diagnostic test OptiMAL-IT (DiaMed AG, Switzerland). All infected patients
577 had *P. falciparum* malaria. The final study population included 41 individuals with malaria
578 and 28 healthy donor controls from the same area. From the malaria-infected cohort, there
579 were 33 men and 8 women, with an average age of 27.1 years (range 16-58 years).

580 Blood samples were centrifuged at 2,400 g for 5 min at room temperature and the
581 plasma was immediately stored at -80°C. Plasma samples were then shipped to the Museum
582 (Paris, France) where they were thawed on ice, aliquoted and stored at -80°C until use.

583 The study was approved by the VietNam People's Army Department of Military
584 Medicine. The purpose of the study was explained to participants in their own language,
585 and oral consent was obtained. Positive patients were treated with dihydroartemisinin-
586 piperaquine combination in accordance with the national drug policy of VietNam.

587

588 **Time-resolved fluoroimmunoassays (TR-FIA)** — TR-FIA assays for hGIIA, hGIIF, hGV
589 and hGX sPLA₂s in plasma were performed as described earlier (27). The assays use rabbit
590 polyclonal anti-sPLA₂ antibodies to set up ELISA-like sandwiches, which were shown to be

591 highly specific for each sPLA₂ isoform (97). hGIII sPLA₂ could not be measured, due to the
592 lack of sensitivity of the corresponding immune serum.

593

594 **Enzymatic assays on *E. coli* membranes** — sPLA₂ activity in plasma was measured by
595 hydrolysis of *E. coli* membranes radiolabeled with [³H]-oleic acid and autoclaved (98). Briefly,
596 40 µL of radiolabeled *E. coli* membranes (100,000 dpm in activity buffer consisting of 0.1 M
597 Tris-HCl pH 8.0, 10 mM CaCl₂, 0.1% BSA) were incubated with plasma (20 µL, diluted 1:150 in
598 activity buffer) at 37°C for 30 minutes. Enzymatic assays were stopped by adding 80 µL of 0.1
599 M EDTA/0.2% fatty acid-free BSA. Reactions were spun down for 5 min at 10,000 g, and the
600 supernatant was collected and counted in a 1450 Microbeta counter (Wallac, Perkin Elmer).

601

602 ***P. falciparum* cultivation in malaria plasma and related assays** — To analyze the anti-
603 *Plasmodium* effect of endogenous sPLA₂ in plasma from infected people, the FcB1 strain was
604 grown in red blood cells of the O- group, brought to 0.1-0.2% parasitaemia, washed and then
605 transferred at 2% haematocrit into basic RPMI supplemented with 2.5 µM hypoxanthine and
606 1 mM CaCl₂ (RPMI-calcium). The cell suspension was distributed into wells of a 96-well
607 microplate (100 µL/well) and then 8 µL of plasma from an infected donor or control normal
608 plasma was added per well. To avoid thermal denaturation of the endogenous sPLA₂ (23, 99),
609 plasma samples were not heat-inactivated prior to parasite cultivation. The microplate was
610 incubated in a candle jar for 96 h, then parasitaemia in each well was determined from Diff-
611 Quik-stained smears.

612 To assess for parasite inhibition by exogenously added hGIIA sPLA₂, experiment was
613 carried out as above, with recombinant hGIIA added at the specified concentration at the
614 start of incubation. To evaluate the contribution of hGIIA catalytic activity, recombinant

615 hGIIA sPLA₂ in its native (WT) or catalytically inactive form (H48Q mutant) (50), and/or the
616 specific hGIIA inhibitor LY311727, were added to the culture at 100 nM (hGIIA WT and
617 H48Q) and 10 µM (LY311727) at the start of incubation.

618

619 ***In vivo* assays with *Plasmodium chabaudi*-infected mice**

620 Mice were housed in the National Museum of Natural History (MNHN) animal facilities
621 accredited by the French Ministry of Agriculture for performing experiments on live rodents.
622 Work on animals was performed in compliance with French and European regulations on
623 care and protection of laboratory animals (EC Directive 2010/63/UE, French Law 2013-118,
624 February 1st, 2013). All experiments were approved by the MNHN's Ethics Committee and
625 registered under the deposit n° APAFIS 201802281454598.

626 C57BL/6J mice (males, 8 to 12-week-old) were inoculated intraperitoneally (IP) with
627 1x10⁶ *P. c. chabaudi* 864VD-infected mouse red blood cells (RBC) in Alsever's solution. Tail
628 blood was taken every 2-3 days within 21 days following inoculation. Parasitaemia was
629 established by optical examination of Diff-Quik-stained blood smears and counting of 2,000
630 RBCs.

631 Parasitized mice were IP-injected with 100 µL of recombinant hGIIA (0.125 mg/kg) in
632 PBS containing 1% mouse serum. Injections were performed twice daily for 3 days, either at
633 the beginning of the patent phase (early injection) or later, i.e. prior to the parasitaemia
634 peak (late injection). Blood parasitaemia was determined every 2-3 days within 22 days
635 following *P. chabaudi* inoculation. Three independent experiments were performed: one
636 early injection experiment with 5 control mice and 6 hGIIA-injected mice, and two late
637 injection experiments, with, respectively, 5 (control) and 7 (injected) mice, and 5 (control)
638 and 6 (injected) mice.

639 To measure plasma peroxidation, mice were sacrificed by exsanguination at day 0
640 before parasite inoculation and at days: 8, 14 and 21 post-inoculation (p.i.). Blood was
641 recovered into EDTA-coated tubes and centrifuged at 800 g for 15 minutes at room
642 temperature. Plasma was frozen at -80°C before TBARS measurement.

643

644 **Statistical analysis** — Data were analyzed using the GraphPad Prism software (San
645 Diego, CA). Normality of groups with n numbers superior to 6 was tested using the Shapiro-
646 Wilk test. When sampling distribution was normal, the parametric unpaired or paired t-test
647 with a two-tailed *P* value was applied. When sampling distribution was not normal and/or
648 the n number was too small (≤ 6), the non-parametric Mann-Whitney U test for independent
649 samples or the Wilcoxon matched-pair test for dependent samples were used. The effect of
650 hGIIA sPLA₂ injection to *P. chabaudi*-infected C57BL/6 mice was analyzed by using the two-
651 way ANOVA test with Bonferroni post-test. Statistical analysis of plasma peroxidation level in
652 the course of infection was performed using the one-way ANOVA with Dunn's multiple
653 comparison test. Differences with *p* values <0.05 (*), <0.01 (**), and <0.001 (***) were
654 considered as statistically significant.

655

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661

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1007 **Legend to figures**

1008

1009 **Figure 1. sPLA₂ enzymatic activity and hGIIA sPLA₂ protein concentration are increased in**
1010 **malaria plasma.** Plasma from 41 *P. falciparum*-parasitized patients (parasitized plasma) and
1011 28 non-parasitized healthy donors (normal plasma) were analyzed for (A) sPLA₂ activity using
1012 the ³H-oleate *E. coli* membrane assay; (B) hGIIA sPLA₂ content by quantitative TR-FIA using
1013 hGIIA-specific antibodies. Median with IQR is shown. The non-parametric Mann-Whitney U
1014 test for independent samples was applied, ****P* < 0.0001. (C): correlation between plasma
1015 hGIIA sPLA₂ concentration and plasma sPLA₂ enzymatic activity (Spearman *r* = 0.9542; ****P* <
1016 0.0001). (D): correlation between plasma hGIIA sPLA₂ concentration and parasitaemia from
1017 donors (Spearman *r* = 0.06894; ns).

1018

1019 **Figure 2: (A) Malaria but not normal plasma spiked with recombinant hGIIA inhibits the**
1020 **growth of *P. falciparum*.** *P. falciparum* was grown for two intraerythrocytic cycles (96 h) in
1021 RPMI containing 8% malaria (M, n=10) or normal plasma (N, n=8) in the absence or pres-
1022 ence (+IIA) of 100 nM recombinant hGIIA sPLA₂. Parasitaemia at time zero and 96 h was
1023 measured from Giemsa-stained smears. Multiplication factor was expressed as Parasitaemia
1024 $t_{96h} / \text{Parasitaemia } t_{0h}$. Paired t test was applied, with **P* < 0.05. (B) hGIIF and hGX sPLA₂s are
1025 **inhibitory to *P. falciparum* in malaria plasma.** Parasites were grown for 96 h in RPMI con-
1026 taining 8% malaria plasma supplemented with either 100 nM hGIIA (IIA), 10 nM hGIIF (IIF),
1027 0.5 nM hGX (X), or without sPLA₂ (w/o). Experiment was performed with 14 (w/o, hGIIA) and
1028 11 (hGIIF, hGX) malaria plasma samples. The nonparametric Wilcoxon signed rank test was
1029 applied for statistical analysis. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

1030

1031 **Figure 3. Phospholipid content and LDL oxidation in malaria plasma.** Plasma samples from
1032 uncomplicated malaria patients (n = 41) and healthy individuals (n = 28 for phospholipid
1033 analysis, n = 19 for oxidation analysis) were analyzed for **(A)** phospholipid content using the
1034 phospholipid B-kit from WAKO Chemicals, and **(B)** lipoprotein oxidation using the Ox-LDL kit
1035 (Merckodia), following manufacturers' instructions. Median with interquartile range (IQR) is
1036 shown. The Mann-Whitney test was applied; *P < 0.05 and ***P < 0.001.

1037

1038 **Figure 4. (A) Exogenous hGIIA sPLA2 hydrolyzes phospholipids from malaria but not**
1039 **normal plasma.** Plasma from parasitized (n = 11, Malaria) and non-parasitized healthy
1040 subjects (n = 10, Normal) were incubated at 37°C for 18 h with 400 nM hGIIA sPLA2 (hGIIA)
1041 or 8 nM hGX sPLA2 (hGX), or without sPLA2 (w/o). NEFA content ($\mu\text{g}/\mu\text{L}$ of plasma) before
1042 and after incubation was measured by using the NEFA-C kit. NEFA release was calculated by
1043 subtracting NEFAs measured at time zero of incubation from NEFAs after 18 h of incubation.
1044 For statistical analysis, the non-parametric Wilcoxon signed rank test was applied. **P < 0.01.

1045 **(B) Parasite inhibition by hGIIA sPLA2 depends on its catalytic activity.** *P. falciparum* was
1046 tested for growth in 7 malaria plasma samples supplemented with 100 nM hGIIA sPLA2,
1047 either WT (WT) or catalytically-inactive (H48Q mutant). Growth control was without sPLA2
1048 (w/o). Multiplication factor after 96 h of incubation in culture conditions was calculated from
1049 parasitaemia at time zero versus 96 h of incubation. For statistical analysis, paired t test was
1050 applied. *P < 0.05, ns = P > 0.05. **(C) The rate of parasite inhibition increases in parallel to**

1051 **the NEFA concentration measured in the supernatant of a culture established in malaria**
1052 **plasma supplemented with recombinant hGIIA sPLA2.** *P. falciparum* was grown for 96 h in
1053 RPMI-calcium with 8% malaria plasma (a mixture of 5 malaria plasma samples)

1054 supplemented with 20, 100 or 200 nM recombinant hGIIA sPLA2. Aliquots were taken every
1055 24 h to determine parasitaemia and measure NEFAs released by hGIIA in the culture
1056 supernatant. Curves: parasite multiplication factor (i.e. % parasitaemia at each time point
1057 divided by % parasitaemia at time zero of incubation) in the presence of 20 (○), 100 (□) and
1058 200 nM hGIIA (■), or its absence (◆). Histograms: NEFAs from the supernatant of the
1059 parasite culture incubated with 20 (white bar), 100 (light grey bar) or 200 nM hGIIA (dark
1060 grey bar). NEFAs at each time point were normalized by subtracting the amount of NEFAs in
1061 the culture supernatant at time zero of incubation.

1062

1063 **Figure 5. Injection of recombinant hGIIA sPLA2 affects Plasmodium development in mice in**
1064 **a time-dependent manner. (A)** Eleven C57BL/6J mice were inoculated at day 0 with *P.*
1065 *chabaudi chabaudi* 864VD. At day 8, 9 and 10 p.i., a group of 6 mice was injected twice daily
1066 by IP with 0.125 mg/kg of recombinant hGIIA sPLA2 (■), while control mice (n=5) were
1067 injected with vehicle (□). **(B)** Twelve mice were infected as in A, then a group of 7 mice were
1068 injected with hGIIA at days 12, 13 and 14 p.i., and control mice (n=5) were injected with
1069 vehicle. Parasitaemia from tail blood was determined at days 6, 9, 12, 13, 14, 16, 19 and 21
1070 p.i. Two-way ANOVA with Bonferroni post-tests was applied for statistical analysis, with
1071 *P<0.05 and ***P<0.001. Arrowheads point out days of injection. **(C)** Plasma peroxidation in
1072 *P. chabaudi*-infected mice is increased at the peak of parasitaemia. Mice from 3 independent
1073 experiments were sacrificed at day 0 or at days 8, 14 and 21 post-inoculation of 1×10^6 RBC
1074 parasitized with *P. chabaudi chabaudi* 864VD. Plasma was analyzed for TBARS content (MDA
1075 equivalent). Values from the 3 experiments have been pooled. One-way ANOVA (Kruskal-
1076 Wallis test) with post-test (Dunn's multiple comparison test) was applied for statistical

1077 analysis; ***P <0.001. Means with SEM are shown. Values from each plasma samples are
1078 presented as dots.

TABLE 1. Specific activities of recombinant human sPLA₂s on lipoproteins.

Specific activities (μmole NEFA / min x mg of sPLA ₂)											
human sPLA ₂	n	Oxidized		Native		Fold-change (oxidized/native)		P values oxidized vs native		P values LDL vs HDL	
		LDL	HDL	LDL	HDL	LDL	HDL	LDL	HDL	native	oxidized
hGIB	4	0.06 ± 0.03	0.45 ± 0.39	0.05 ± 0.02	0.25 ± 0.22	1.2	1.8	0.1133	0.1250	0.0625	0.0625
hGIIA	7	0.16 ± 0.09	0.10 ± 0.06	0.02 ± 0.02	0.03 ± 0.03	8.0	3.3	0.0156*	0.0313*	0.5982	0.0156 *
hGIID	2	0.04	0.03	0.03	0.04	-	-	-	-	-	-
hGIIIE	2	0.03	0.02	0.02	0.02	-	-	-	-	-	-
hGIIF	6	1.04 ± 0.28	1.56 ± 0.64	0.74 ± 0.21	1.58 ± 0.59	1.4	1.0	0.0313*	0.8438	0.0139*	0.1563
hGIII	4	4.29 ± 3.84	0.82 ± 0.51	6.29 ± 5.37	1.06 ± 0.70	0.7	0.7	0.2297	0.0961	0.0447*	0.0931
hGV	6	0.99 ± 0.37	3.27 ± 2.57	1.05 ± 0.44	3.88 ± 2.20	0.9	0.8	0.8438	0.1563	0.0313*	0.0625
hGX	7	2.66 ± 1.27	5.31 ± 2.12	2.58 ± 1.60	7.92 ± 5.08	1.0	0.6	0.6875	0.1094	0.2188	0.0313 *
hGXIIA	2	< 0.01	< 0.01	< 0.01	< 0.01	-	-	-	-	-	-

Values are mean ± SD (μmole NEFA/min x mg of sPLA₂). For statistical analysis, the non-parametric Wilcoxon matched-pair test was used. **P* <0.05. n, number of independent experiments. (-): not determined.

TABLE 2. Anti-*P. falciparum* activities of lipoproteins after hydrolysis by human sPLA₂s.

	Oxidized lipoproteins		<i>P</i> value	Native lipoproteins		<i>P</i> value	
	IC ₅₀ (µg PC/mL)			IC ₅₀ (µg PC/mL)			
Pretreatment with 20 nM sPLA ₂	LDL	HDL	Oxidized LDL vs oxidized HDL	LDL	HDL	LDL (oxidized vs native)	HDL (oxidized vs native)
none	220.1 ± 61.7	> 250.0	-	> 250.0	> 250.0	-	-
hGIB	207.5 ± 59.5	185.2 ± 56.6	-	> 250.0	> 250.0	-	-
hGIIA	220.7 ± 59.3	> 250.0	-	> 250.0	> 250.0	-	-
hGIID	222.3 ± 60.6	> 250.0	-	226.6 ± 40.7	> 250.0	-	-
hGIIIE	224.7 ± 54.8	> 250.0	-	> 250.0	238.7 ± 43.3	-	-
hGIIF	151.4 ± 49.7	147.6 ± 49.7	0.8447	190.0 ± 89.5	157.4 ± 68.9	0.3970	0.4375
hGIII	166.4 ± 63.1	196.8 ± 77.5	0.0209*	115.0 ± 43.9	210.2 ± 99.9	0.0498*	0.6747
hGV	195.6 ± 67.9	141.6 ± 45.5	0.0089**	241.6 ± 133.8	181.2 ± 84.5	0.2604	0.1198
hGX	101.8 ± 16.5	75.6 ± 11.2	0.0355*	111.6 ± 37.3	81.8 ± 13.3	0.5155	0.4294
hGXIIA	228.2 ± 54.1	>250.0	-	> 250.0	>250.0	-	-

Values are mean ± SD of 5 independent experiments. For statistical analysis, the nonparametric Wilcoxon matched pairs test was applied, with: **P* < 0.05 and ***P* < 0.01 considered significant. (-): not determined.

TABLE 3. Anti-*P. falciparum* activities of lipoproteins after hydrolysis with high concentrations of hGIIA sPLA₂

Anti-<i>Plasmodium</i> activities of LDL and HDL				
IC ₅₀ values (μg PC/mL)				
Pretreatment with hGIIA sPLA₂ (nM)	Native LDL	Oxidized LDL	Native HDL	Oxidized HDL
None	> 250	220.1 ± 61.7	> 250	> 250
100	± 250	162.3 ± 22.4	> 250	> 250
250	215.3 ± 26.4	87.0 ± 39.2	216.6 ± 23.5	87.3 ± 9.2

Values are mean ± SD of 3 independent experiments

Table 4. Anti-*P. falciparum* activities of human sPLA₂s in the presence of native or oxidized lipoprotein fraction from human plasma.

Anti-<i>P. falciparum</i> activities of human sPLA₂s				
IC ₅₀ values (nM)				
Total lipoproteins	hGIIA	hGIIF	hGV	hGX
w/o	> 250.0	> 50.0	> 80.0	> 3.0
Native	> 250.0	19.8 ± 13.3	21.9 ± 3.3	1.52 ± 0.74
Oxidized	150.0 ± 40.8	4.3 ± 4.0	10.2 ± 2.7	0.78 ± 0.26

Values are mean ± SD of 4 independent parasite growth inhibition assays. The lipoprotein fraction was 0.2 mg PC /mL in culture medium.

Table 5: *In vitro* anti-*Plasmodium* effect of native and oxidized PUFAs

Anti-<i>P. falciparum</i> activities of native and oxidized PUFAs			
IC ₅₀ values (μM)			
	Native	Oxidized	P value (native vs oxidized)
Arachidonic acid (C20:4, n-6)	10.6 ± 2.1	16.1 ± 2.7	0.1293
Eicosapentaenoic acid (C20:5, n-3)	7.4 ± 1.4	25.9 ± 5.1	0.0030**
Docosahexaenoic acid (C22:6, n-3)	10.5 ± 1.0	65.3 ± 23.2	0.0315*

IC₅₀ values are mean ± SEM of 3 independent experiments in triplicate. Unpaired t test was applied for statistical analysis, with *P <0.05 and **P <0.01 considered significant.

FIGURE 1

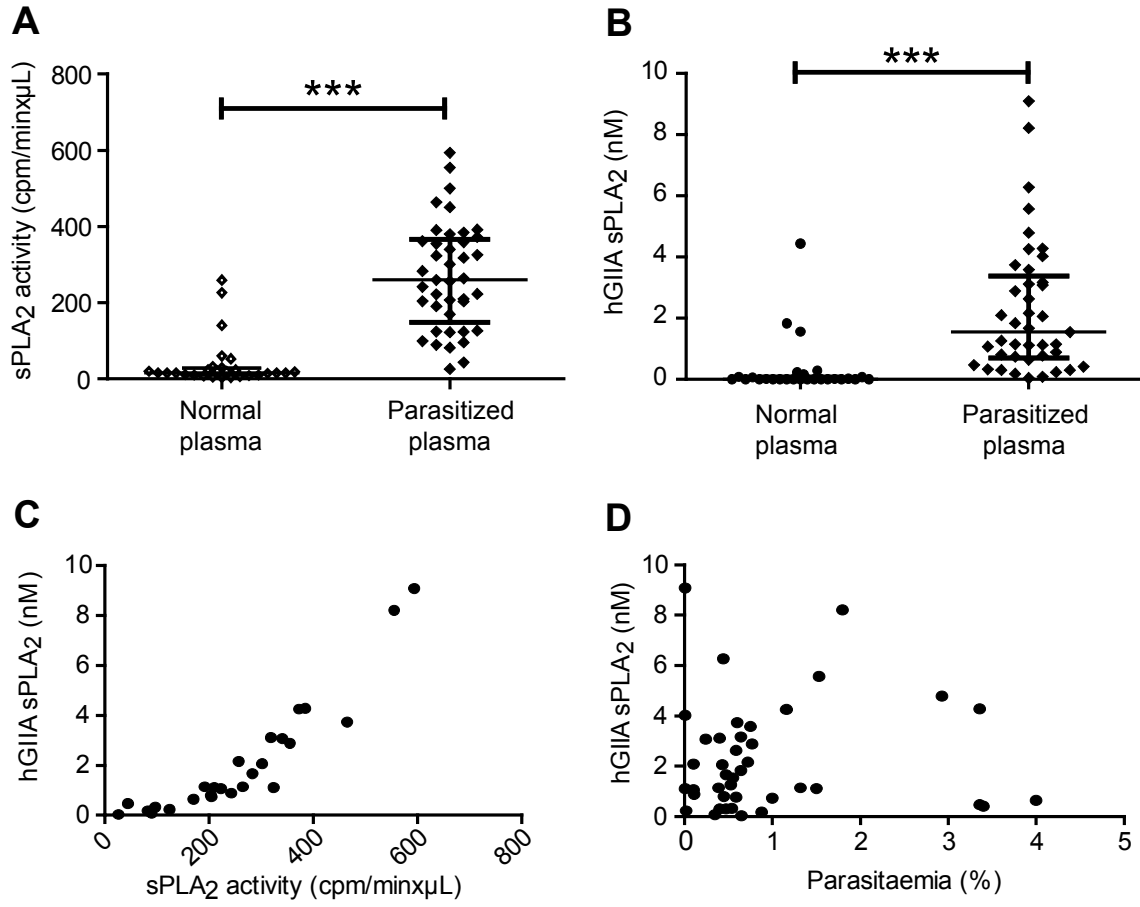


FIGURE 2

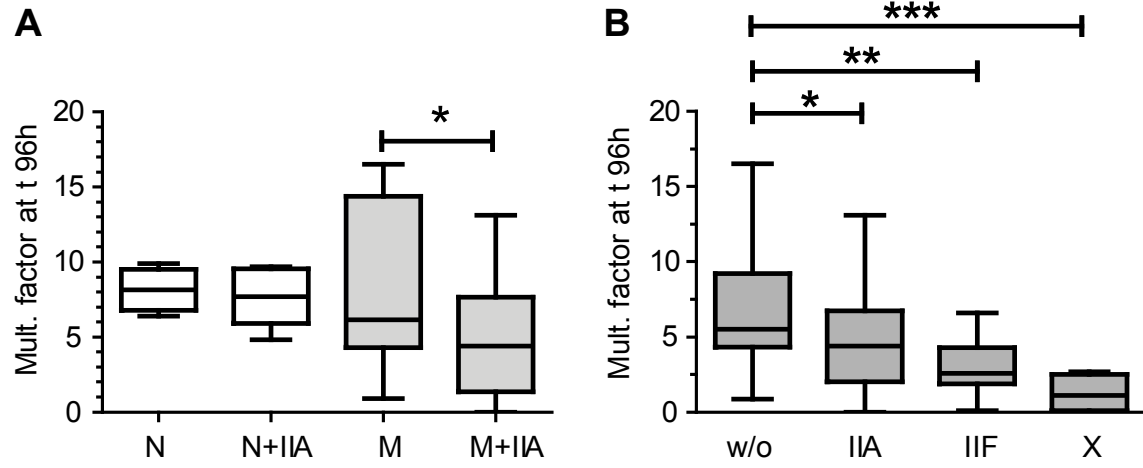


FIGURE 3

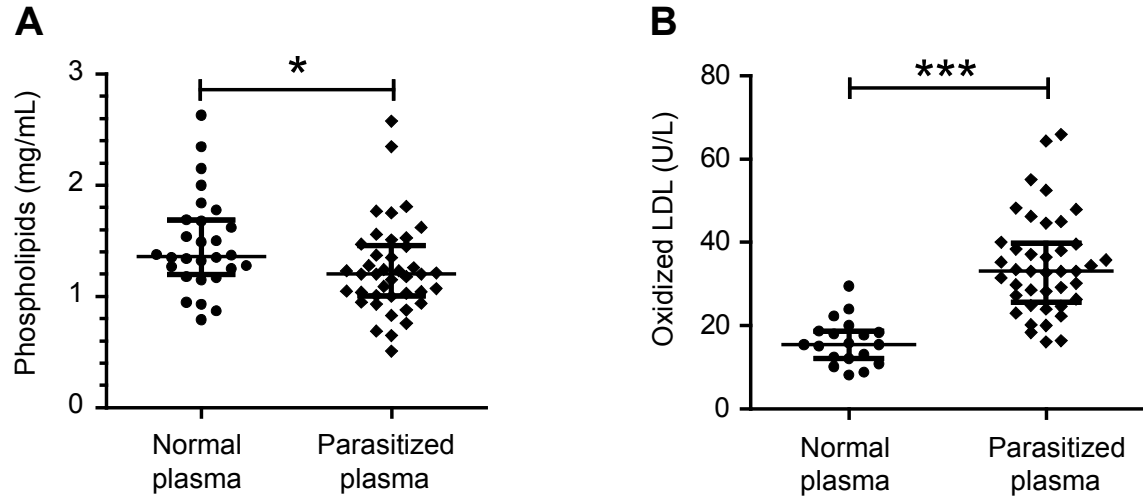


FIGURE 4

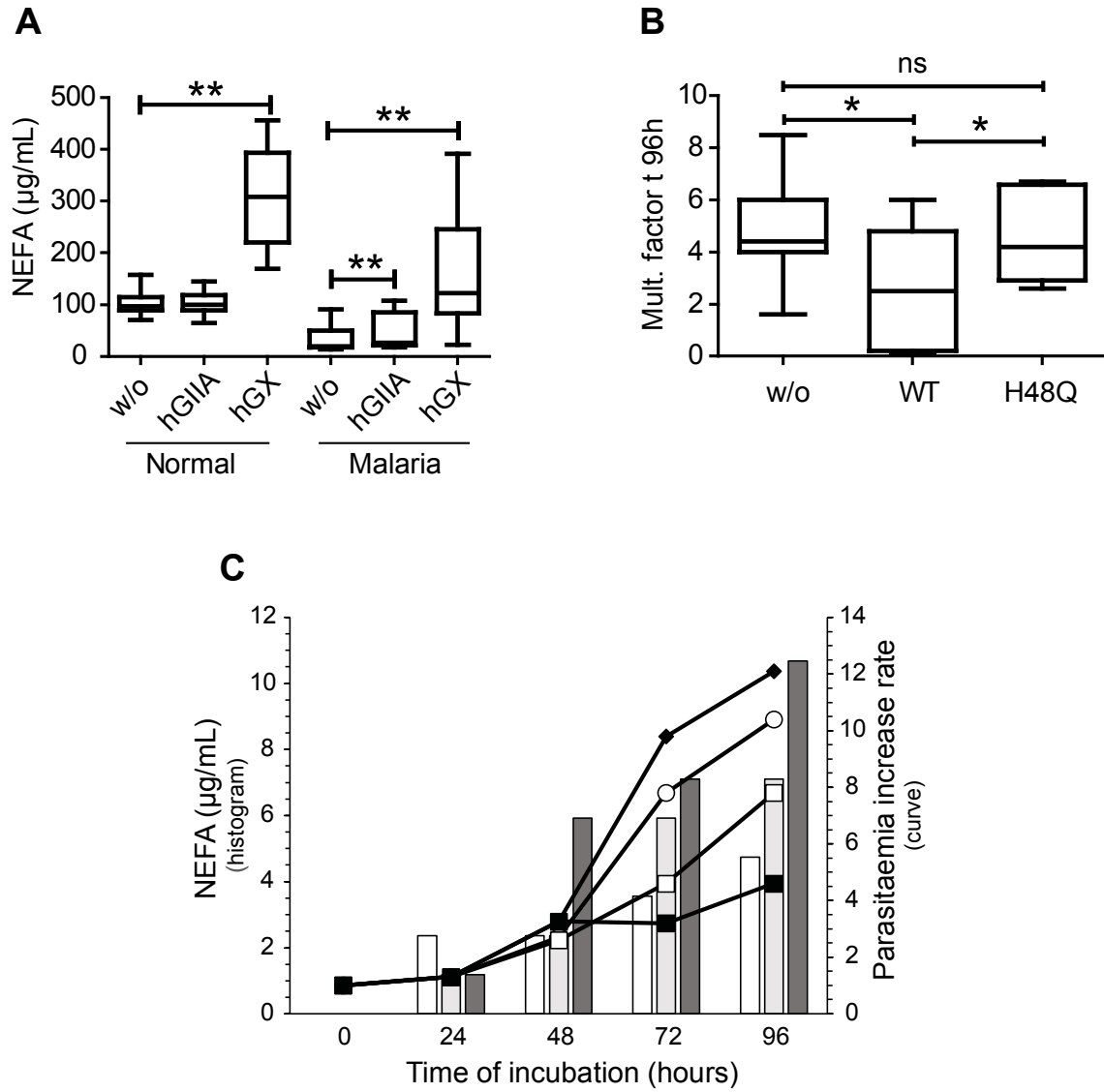


FIGURE 5

