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1 **HLA-G expression during hookworm infection in pregnant women**

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19 **Introduction:** HLA-G plays a key role on immune tolerance. Pathogens can induce soluble  
20 HLA-G (sHLA-G) production to down-regulate the host immune response, creating a  
21 tolerogenic environment favorable for their dissemination. To our knowledge, no study has  
22 yet been conducted to assess the relationship between sHLA-G and geohelminth infections.

23 **Methods:** The study was conducted in Allada, Southeastern Benin, from 2011-2014. The  
24 study population encompassed 400 pregnant women, included before the end of the 28<sup>th</sup>  
25 week of gestation and followed-up until delivery. At two antenatal care visits and at delivery,  
26 stool and blood samples were collected. Helminths were diagnosed by means of the Kato-  
27 Katz concentration technique. We used quantile regression to analyze the association  
28 between helminth infections and sHLA-G levels during pregnancy.

29 **Results:** sHLA-G levels gradually increased during pregnancy and reached maximal levels  
30 at delivery. Prevalence of helminth infections was low, with a majority of hookworm  
31 infections. We found significantly more hookworm-infected women above the 80<sup>th</sup> quantile  
32 (Q80) of the distribution of the mean sHLA-G level ( $p < 0.03$ , multivariate quantile regression).  
33 Considering only women above the Q80 percentile, the mean sHLA-G level was significantly  
34 higher in hookworm-infected compared to uninfected women ( $p = 0.04$ ).

35 **Conclusion:** High levels of sHLA-G were associated with hookworm infection in pregnant  
36 women. This result is consistent with the potential involvement of sHLA-G in immune  
37 tolerance induced by helminths during pregnancy.

38

## 39 1. Introduction

40 Helminths, and particularly geohelminths, infect 1.45 billion people worldwide (Pullan et al.,  
41 2014). The prevalence of geohelminth infection during pregnancy in endemic African areas  
42 could reach more than 70% (van Eijk et al., 2009). Children born to infected mothers are  
43 reported to be more susceptible to helminth infection and to display weaker responses to  
44 vaccines (Cooper et al., 1998; Elias et al., 2005; Malhotra et al., 1999; Malhotra et al., 2003;  
45 Mehta et al., 2012; Sabin et al., 1996). These last observations could be explained by the  
46 phenomenon of immune tolerance. The latter was also suspected in children exposed to  
47 malaria *in utero* (Malhotra et al., 2009). Lymphocyte responses to parasite antigens and  
48 detection of specific immunoglobulins in cord blood, consistent with *in utero* sensitization,  
49 have been described for several helminth species including ascariasis and hookworm  
50 (Guadalupe et al., 2009; Larocque et al., 2006; Pit et al., 2000). Considering that the human  
51 leukocyte antigen-G (HLA-G) is a well-recognized immune checkpoint molecule, the study of  
52 the potential involvement of this molecule in geohelminth infections is of interest.

53 Human Leukocyte Antigen-G (HLA-G) is a non-classical HLA class I antigen that differs from  
54 classical class I molecules by its restricted tissue distribution, diversity of protein isoforms  
55 and limited gene polymorphism (Donadi et al., 2011). Seven isoforms of the protein are  
56 generated by alternative splicing, four are membrane bound (HLA-G1-4) and three are  
57 soluble (HLA-G5-7 of which the shed HLA-G1 and the soluble HLA-G5 are usually measured  
58 in plasma samples). HLA-G further differs from classical class I molecules through its  
59 immune regulatory functions, which is of paramount importance in maternal-fetal immune  
60 tolerance by inhibiting the cytotoxic effect of maternal NK cells (Moreau et al., 1998; Rouas-  
61 Freiss et al., 1997). A lack or a failure of HLA-G expression may lead to a breach of fetal-  
62 maternal tolerance and consequent fetus rejection (Cecati et al., 2011; Papamitsou et al.,  
63 2014). Modulation of HLA-G expression has also been described in tumor cell development,  
64 allogenic organ engraftment (Carosella et al., 2015) and viral infections (Amiot et al., 2014b).  
65 It has been suggested that high HLA-G levels induce tolerance and contribute to immune

66 evasion by tumor cells (Morandi et al., 2016; Pistoia et al., 2007) or viruses (Amiot et al.,  
67 2014a; Amiot et al., 2014b).

68 In tropical areas, parasitic infections during pregnancy modify the immune development of  
69 the fetus (Dauby et al., 2012). Such infections affect the susceptibility of young children to  
70 infectious pathogens, their response to vaccines, and the development of allergic disorders  
71 such as asthma and rhinitis (Thornton et al., 2010). Placental *Plasmodium falciparum*  
72 infection is associated with susceptibility to malaria during infancy (Bouaziz et al., 2017; Le  
73 Hesran et al., 1997; Le Port et al., 2011; Mutabingwa et al., 2005; Schwarz et al., 2008) and it  
74 has been hypothesized that immune tolerance may be responsible for the shortening of time  
75 to the first occurrence of malaria infection, but a complete explanation of the physiopathology  
76 involved has yet to be established (Moya-Alvarez et al., 2014). Furthermore, placental  
77 malaria is also associated with non-malarial infections, suggesting that immune tolerance  
78 may go beyond malaria-specific immunity (Rachas et al., 2012). The association of HLA-G  
79 with the susceptibility to malaria during infancy and with low birth weight could reflect the  
80 potential involvement of sHLA-G in immune tolerance mediated by placental malaria  
81 (d'Almeida et al., 2016; d'Almeida et al., 2017; Sadissou et al., 2014).

82 Considering that the expression of HLA-G may modulate viral and parasitic infections  
83 (Sabbagh et al., 2018), and considering that HLA-G plays an important role on pregnancy, in  
84 the present study we investigated the potential association between soluble HLA-G levels  
85 and hookworm infections during pregnancy.

## 86 **2. Material and methods**

### 87 **2.1. Study area, study design and follow-up**

88 Our study took place in Allada, a town located 56 km North Cotonou, the economic capital of  
89 Benin. The study participants were residents of 21 villages surrounding two primary health  
90 facilities: Sékou and Attogon. This study is part of a larger one encompassing 1,183 human  
91 immunodeficiency virus (HIV)-negative pregnant women participating in the randomized

92 Malaria in Pregnancy Preventive Alternative Drugs ([http://clinicaltrials.gov/ct2/show/](http://clinicaltrials.gov/ct2/show/NCT00811421)  
93 NCT00811421) trial, designed to compare intermittent preventive malarial treatment (IPTp)  
94 with either sulfadoxine-pyrimethamine or mefloquine. The study protocol has been described  
95 in detail elsewhere (Accrombessi et al., 2015; Ouedraogo et al., 2012). The first 400 children  
96 of these women were followed-up to investigate the impact of sociodemographic, economic,  
97 environmental and biologic factors such as the parasitological status (geohelminth and *P.*  
98 *falciparum* carriers) during pregnancy on infant health during the two years of life in southern  
99 Benin (Accrombessi et al., 2015; d'Almeida et al., 2017). The 400 mothers included in the  
100 present study are the mothers of these 400 children. Women were included before a  
101 gestational age (GA) of 28 weeks, and two doses of IPTp were administered at antenatal  
102 visits (ANV). The second dose was given at least 1 month after the first one. During the first  
103 ANV, a total dose of 600 mg of mebendazole was given to the women to be taken at home  
104 (100 mg two times per day for 3 days) according to the guidelines of the Beninese Ministry of  
105 Health. Between antenatal visits, women were encouraged to attend the health facility for  
106 any health complaint. At inclusion, a questionnaire was applied by the midwife to obtain data  
107 regarding socio-demographic characteristics, gynecological and obstetrical history. During  
108 ANV1 and ANV2 (for IPTp1 and IPTp2, respectively) and at delivery, blood and stool  
109 samples were collected before the intake of IPTp, to allow plasma sHLA-G measurements as  
110 well as malaria and geohelminth diagnoses. At delivery, a placental blood smear was  
111 collected to detect placental malaria. Malarial infection was defined as the presence of  
112 asexual *P. falciparum* parasites in a blood smear. All the medications prescribed during the  
113 follow-up were free of charge.

#### 114 **Soluble HLA-G quantification**

115 Soluble HLA-G (sHLA-G) was quantified using MEM-G/9 antibody, which recognizes  
116 the most abundant soluble isoforms (shed sHLA-G1 and intron 4 containing secreted HLA-  
117 G5), and an anti-human  $\beta$ 2-microglobulin antibody, respectively, as capture and detection  
118 antibodies (Menier et al., 2003; Rebmann et al., 2005). Microtitration plates were coated with

119 10 µg/mL MEM-G/9 mouse-anti-human HLA-G mAb (Exbio, Praha, Czech Republic) and  
120 incubated overnight at 4°C. Plates were saturated with 300 µL ready to use diluent buffer  
121 (DAKO, Carpinteria, CA, USA) for 2 hours. Plasma samples were diluted ( $\frac{1}{2}$ ) in diluent  
122 buffer, tested in duplicate and incubated for 2 hours. After incubation and washing,  
123 monoclonal anti-human beta2-microglobulin antibody (DAKO, Glostrup, Denmark), which  
124 recognizes the immobilized antibody sHLA-G complex, was added to the wells and incubated  
125 for 1 hour. The plates were then incubated for 1 hour with 100 µL (1:200) envision buffer +  
126 system HRP (DAKO, Carpinteria, CA, USA) to obtain anti-β2-microglobulin-horseradish  
127 peroxidase complex to improve the efficiency of the reaction. All incubation steps were  
128 performed at room temperature and followed by four washes using washing buffer (H<sub>2</sub>O,  
129 PBS 1X, 0.1% Tween 20). The plates were incubated for 30 min with substrate  
130 (Tetramethylbenzidine super sensitive-TMB, Sigma Aldrich, Saint Louis, MO, USA) and  
131 absorbance was measured at 450 nm after adding HCL (1 N). Total sHLA-G levels were  
132 determined from a five-point standard calibration curve using dilutions (12.5- 200 ng/mL) of  
133 HLA-G5 purified from M8-HLA-G5 cell line culture supernatants. Results are presented in  
134 ng/mL.

### 135 **Helminth identification**

136 Women were invited to provide a sample of their fresh morning stool put in a 120 mL plastic  
137 container that was distributed beforehand. Stool samples were transferred to the health  
138 centre laboratory and were analysed at the same day, using standard 41.7 mg plastic  
139 templates, and thick smears were examined under a light microscope by experienced  
140 laboratory technicians. Microscopical identification of *Ascaris lumbricoides* and *Trichuris*  
141 *trichiura* eggs is easy recognizable using the Kato-Katz method; however, *Ancylostoma*  
142 *duodenale* and *Necator americanus* eggs cannot be distinguished. For this reason, we  
143 included both *Ancylostoma duodenale* and *Necator americanus* species in a same hookworm  
144 group.

145 **Ethics**

146 The protocol received approval from the Comité Consultatif de Déontologie et d’Ethique of  
147 the Institut de Recherche pour le Développement (IRD, France) and from the Ethics  
148 Committee of the Faculté des Sciences de la Santé of Cotonou  
149 (N°43/11/2010/CE/FSS/UAC). Before inclusion, the protocol of the study was explained in the  
150 relevant local languages and written consent was obtained. Women who could not read and  
151 write provided thumbprints to confirm their agreement to participate in the study after a nurse  
152 had explained the study.

153 **Definition and characteristics of the study variables**

154 **Soluble HLA-G levels**

155 The outcome variable is the level of sHLA-G, used as a quantitative variable after a  
156 logarithmic transformation to take into account the skewness of the distribution.

157 **Variable of interest: Helminth infections**

158 Both hookworms (*Ancylostoma duodenale*, *Necator americanus*) and round worms (*Ascaris*  
159 *lumbricoides* and *Trichuris trichiura*) were detected in faecal samples. However, due to the  
160 very low prevalence of both *Ascaris lumbricoides* and *Trichuris trichiura*, our analyses  
161 focused on hookworm infection during pregnancy, by creating a variable coded 1 if at least  
162 one hookworm egg was detected during follow-up.

163 **Covariates**

164 Information on the following variables was collected: gravidity, IPTp regimen, placental  
165 malaria, inclusion health center, ethnicity, marital status, occupation, education level, latrine  
166 use at home and “wealth score”. The wealth score was computed using four socio-economic  
167 items which were theoretically associated with social welfare: motorbike, television,  
168 electricity, and fridge. Each item could take the value 0 or 1 (yes = 1, no = 0), and the score  
169 was obtained by summing the values of each item.

## 170 **Statistical analysis**

171 Categorical variables were described using proportions and 95% confidence interval and the  
172 continuous variables were described by the median and interquartile range. To compare  
173 proportions, we used the Chi-square, Fisher T and Cuzick trend tests. Continuous variables  
174 were compared by means of Student T test, Wilcoxon-Mann-Witney rank-sum test and  
175 analysis of variance (ANOVA). We checked for normality of distribution using  
176 Skewness/Kurtosis normality test and compared the variances with Breusch-Pagan/Cook-  
177 Weisberg heteroscedasticity test. A p-value less than 5% was considered as significant.

178 Classical methods for repeated measurements (mixed models) were not used to analyse  
179 sHLA-G level at antenatal visits because each antenatal visit had its particular characteristics  
180 and cannot be considered as a repeated measurement of the same variable under the same  
181 conditions. Measurements at the first antenatal visit were done before any intervention.  
182 Medication was given at ANV1 and ANV2 (IPTp for malaria during the two first antenatal  
183 visits and mebendazole during the first one). Furthermore, the third measurement occurred at  
184 delivery. Consequently, to create a unique variable accounting for the level of sHLA-G during  
185 pregnancy, a mean sHLA-G (MsHLA-G) level was computed. To take into account the fact  
186 that not all women were present at each measurement time, and that sHLA-G levels  
187 increased significantly at delivery, the MsHLA-G was computed for each woman as the mean  
188 of her visit-adjusted sHLA-G level. The visit-adjusted sHLA-G levels were obtained by  
189 subtracting the mean sHLA-G of the population at each visit. The association between  
190 MsHLA-G and hookworm infection was analyzed by means of quantile regression that takes  
191 into account the heteroscedasticity of the distribution (Koenker, 2005; Sims, 1996). Univariate  
192 and multivariate analyses were done and variables with p-value less than 0.2 in univariate  
193 analysis were considered in the multivariate model. The variable of interest was “at least one  
194 hookworm infection during the follow-up”. Hookworm and “inclusion health facility” were  
195 included in the multivariate analysis. The other covariates considered were: gravidity,  
196 regimen of intermittent preventive treatment, placental malaria, age, ethnicity, marital status,  
197 occupation, education, latrines use at home and wealth score. Starting from the initial model

198 we proceeded using a step-by-step backward regression. Only the variables exhibiting p-  
199 value<0.05 were maintained in the final model. All analyses were performed using Stata12  
200 software (StatCorp LP, College Station, TX, USA).

## 201 **Results**

### 202 **Study population**

203 Four hundred pregnant women were included in the study. Their median age was 25 years  
204 (range: 18-42; Interquartile range [IQR]: 21-30). The median gestational age (GA) at inclusion  
205 was 24 weeks (range: 13-28; IQR: 20-26). The median wealth score based on 4 variables  
206 (ownership of motorbike, electricity, television, and refrigerator) was 1, (range: 0-4, IQR: 1-2).  
207 The socio-demographic and economic characteristics of the participants are summarized in  
208 Table 1.

### 209 **Helminth infections**

210 The results of parasitic coprology are shown in Figure 1. Stool samples were collected from  
211 395 (98.75%) participants at ANV1, 387 (96.75%) at ANV2 and 355 (88.75%) at delivery,  
212 giving a total of 1137 stool samples collected. Among the 1137 stool samples, helminth eggs  
213 were detected in 88 stool samples (7.7%). The sample positivity rate to helminth infections  
214 was 9% at ANV1, 8.8% at ANV2 and 5% at delivery. Hookworm eggs (*Ancylostoma*  
215 *duodenale* and *Necator americanus*) were found in 74 samples (27 infections at ANV1, 31 at  
216 ANV2 and 16 at delivery), 10 samples contained eggs of *Ascaris lumbricoides* (1 at ANV1, 6  
217 at ANV2 and 3 at delivery) and eggs of *Trichuris trichiura* were found in 3 stool samples (1  
218 sample positive at ANV1, 1 at ANV2 and 1 at delivery, all from different participants).

219 The prevalence of helminth infection (at least one geohelminth in the stools during the whole  
220 follow-up) in the study population was 18.5% (74/400). The majority of the helminth infections  
221 detected in the stools were hookworm infections (86.5% (64/74)), while *Ascaris lumbricoides*  
222 and *Trichuris trichiura* eggs were respectively detected in 13.5% (10/74) and 4% (3/74) of the

223 positive stool samples. Two participants were co-infected with both hookworm and *Ascaris*  
224 *lumbricoides*, one diagnosed at ANV2 and the other diagnosed at delivery.

225 The distribution of hookworm infection according to the different characteristics of the study  
226 population is presented in Table 2. The prevalence of hookworm infection was significantly  
227 higher in Sekou health facility and in the Aizo ethnic group ( $p=0.02$  in both cases,  $\text{Chi}^2$ ). The  
228 possession of latrines was associated with a significantly lower prevalence ( $p=0.001$ ,  $\text{Chi}^2$ ) as  
229 was a high wealth score ( $p=0.024$ ,  $\text{Chi}^2$ ).

### 230 **Soluble HLA-G level**

231 Soluble HLA-G measurement was available on three occasions from 85% of mothers and  
232 98% were sampled at least twice. sHLA-G levels were stable over the two first visits (mean  
233 sHLA-G level at ANV1 = 10.09 ng/ml and mean sHLA-G level at ANV2 = 10.57 ng/ $\mu$ l) but  
234 increased significantly at delivery (mean sHLA-G level = 17.26 ng/ $\mu$ l ( $p<10^{-3}$ , ANOVA)).

### 235 **Relation between hookworm infection and adjusted mean of sHLA-G.**

236 The distribution of adjusted means of sHLA-G (MsHLA-G) is presented in Figure 2. Despite  
237 the log-transformation, both the hypotheses of normality ( $p<0.0001$ , Shapiro Wilk normality  
238 test) and of homoscedasticity ( $p=0.02$ ; Breusch-Pagan/Cook-Weisberg heteroscedasticity  
239 test) were significantly rejected. To deal with both the skewed distribution and  
240 heteroscedasticity, a quantile (5<sup>th</sup>, 10<sup>th</sup>, up to 95<sup>th</sup> quantile) regression (Chamberlain, 1994)  
241 was used to analyze the effect of hookworm infection and of other covariates (inclusion  
242 health facility, latrines use at home, wealth score, gravidity, regimen of intermittent preventive  
243 treatment, placental malaria, age, ethnicity, marital status, occupation, education) on the  
244 different quantiles of mean sHLA-G levels.

245 In univariate analysis, inclusion health facility had a significant effect on the distribution of  
246 sHLA-G with significantly fewer women above the Q50 of the distribution of the mean level of  
247 sHLA-G included at Sekou health center (Table 3). No significant effect of the others  
248 covariates was detected on the distribution of sHLA-G. As hookworm infection is the variable

249 of interest, the multivariate model of regression included the inclusion health center and  
250 hookworm infection. The effect of inclusion health facility on the distribution of sHLA-G  
251 remained significant in multivariate analysis and a significant effect of hookworm infection  
252 was observed ( $p < 0.03$ , multiple quantile regression). Significantly more hookworm-infected  
253 women were found above the Q80 percentile of sHLA-G distribution (Table 3 and [Figure 3](#)).  
254 Considering only women above the Q80 percentile, the geometric mean level of sHLA-G was  
255 significantly higher in hookworm-infected than in uninfected women (1.57 vs 1.41  
256 respectively;  $p = 0.04$ , Student T test).

## 257 **Discussion**

258 The main results of the present work show a relatively low frequency of helminth infection  
259 during pregnancy of Beninese women; however, we observed an association between the  
260 plasma levels of the tolerogenic molecule HLA-G and hookworm infection.

261 Prevalence of hookworm infections in our study decreased from 9% at ANV1 to 5% at  
262 delivery. The detection of hookworms was performed with the inexpensive and field-  
263 applicable Kato-Katz method however the low sensibility of the Kato-Katz method probably  
264 underestimates the intestinal helminth infection prevalence in our study population.

265 This finding could be explained by the intake of one dose of mebendazole (600 mg) during  
266 antenatal visits after 16<sup>th</sup> week of gestation, as recommended by the Beninese National  
267 Health Program. Anthelmintic treatment during pregnancy aims to fight against maternal  
268 anemia (Ouedraogo et al., 2012), which has an important impact on neonatal and infant  
269 health (Koura et al., 2012). Helminth infections during pregnancy are also associated with  
270 increased susceptibility to geohelminth infections in the children (Mehta et al., 2012) and to  
271 weaker anti-vaccine responses (Labeaud et al., 2009). The mechanisms by which maternal  
272 helminth infection can influence immune development of the fetus are partially understood  
273 (Mpairwe et al., 2014). However, epidemiological evidence suggests that *in utero*  
274 sensitization results in down-regulation of immune responses in offspring (Dauby et al.,

275 2012), leading to a phenomenon of immune tolerance, in which HLA-G could play a crucial  
276 role.

277 In the multivariate model of regression, sHLA-G levels were associated with health center  
278 and hookworm infection. Women included at Sekou health center had lower level of sHLA-G  
279 than those from Attogon health center. Significant differences in term of ethnicity group  
280 distribution and wealth score were observed between both health centers ( $p < 0.001$  and  
281  $p < 0.001$  respectively, Chi-Square test). Women from Fon ethnicity group were more  
282 represented at Attogon than at Sekou (57 % versus 9 %) while women from Aizo ethnicity  
283 group were more frequent at Sekou than Attogon (82 % versus 31 %). Differences in  
284 behaviour and/or in genetic mechanism influencing regulation of HLA-G expression between  
285 both ethnicity groups could partly explain the modulation of sHLA-G observed between  
286 health centers. It is important to emphasize that Sekou is a semi-rural village while Attogon is  
287 more rural therefore difference of HLA-G expression could also be related to environmental  
288 factors, such as the prevalence of other pathogens that could also influence the expression  
289 of sHLA-G. The elevated risk of exposure to mosquito bites was previously associated with  
290 an increased expression of sHLA-G (Sadissou et al., 2014). In the present work, no  
291 association was observed between peripheral or placental malaria infections during  
292 pregnancy and a significant modulation of plasmatic sHLA-G in mothers (d'Almeida et al.,  
293 2017). Difference of sHLA-G level observed between the health centers seems not directly  
294 related to malaria infection.

295 Our results show that there are more hookworm infected women above the Q80 and that  
296 these women have significantly higher levels of sHLA-G than not infected women from the  
297 same quantiles. The use of Kato-Katz is a potential limitation in our study. Some intestinal  
298 helminth infections could be failed in case of light-intensity hookworm infections and we have  
299 potentially included false negatives in the group of "hookworm uninfected women". Such  
300 misclassification should not significantly affect our result because a higher level of sHLA-G is  
301 expected in presence of false positives in the group of women with no hookworm egg. Such  
302 situation of misclassification could typically lead to a lack of statistical power, which could

303 reduce the chance to find an association between hookworm infection and sHLA-G levels.  
304 The use of more sensitive tools such as the PCR or coproculture methods could reinforce the  
305 result of association between hookworm infection and sHLA-G levels obtained in our study  
306 and give a better insight of the hookworm prevalence in the study areas.

307 It is known that hookworm infection results firstly in a T helper 2 cell type polarization,  
308 characterized by the proliferation of T cells that produce IL-4, IL-5 and IL-13, activation of  
309 basophils, eosinophils, mast cells and secretion of high levels of IgE. Then, a progressive  
310 and generalized immunosuppression, characterized by induction of regulatory responses  
311 (regulatory T, B and dendritic cells) with increased expression of the regulatory cytokines IL-  
312 10 and TGF- $\beta$ , often occurs. The progressive emergence of an immunosuppressive state  
313 could be a mechanism by which hookworms escape the effective anti-helminthic Th2  
314 response (Borkow et al., 2000; Maizels et al., 2004). HLA-G can participate in this  
315 immunoregulatory state (Carosella et al., 2015) that can lead to a tolerogenic environment.

316 HLA-G is a very complex molecule with well-recognized biological features, and its immune  
317 functions have recently been reviewed (Carosella et al., 2015). HLA-G is known to inhibit the  
318 function of T, NK and B cells through direct interaction with ILT2. Recently, Naji et al., (2014)  
319 showed that HLA-G inhibits B cell proliferation, differentiation, and immunoglobulin secretion  
320 in both T cell–dependent and T cell–independent models of B cell activation (Naji et al.,  
321 2014). It has been recently shown that children born to mother with high sHLA-G levels are  
322 predisposed to have high sHLA-G levels during their two first years of life (d'Almeida et al.,  
323 2016). We can then hypothesise that the high levels of sHLA-G found in hookworm infected  
324 women could be associated with high levels in their newborns that can be involved in the  
325 immune tolerance phenomenon.

326 In tropical areas, immune tolerance is not restricted to helminth infections (Raberg, 2014).  
327 Placental malaria is classically described as a risk factor for malaria in early infancy (Le  
328 Hesran et al., 1997; Le Port et al., 2011; Malhotra et al., 2009; Mutabingwa et al., 2005) and  
329 although not totally understood, induction of a tolerant state has also been proposed to be an  
330 explanation to this susceptibility. However, we have recently demonstrated that children born

331 to mothers with placental malaria are not only more susceptible to malaria but also to non-  
332 malarial fever (Rachas et al., 2012). These findings underline the fact that immune tolerance  
333 could impact on immunity in a more general manner rather than simply malaria-specific  
334 immunity. The involvement of HLA-G in tropical diseases has been recently shown for both  
335 malaria and human African trypanosomiasis at both genetic and protein levels (Courtin et al.,  
336 2013; d'Almeida et al., 2016; Garcia et al., 2013; Gineau et al., 2016; Sadissou et al., 2014).  
337 Some associations between antibody responses to malarial antigens and some  
338 polymorphisms of *HLA-G* strengthen the concept of a potential role of HLA-G in the control of  
339 host immunity in tropical diseases (Sabbagh et al., 2013).

340 Our results also showed a significant increase of sHLA-G levels at delivery compared to the  
341 sHLA-G levels quantified during antenatal visits. Similar result was previously observed in a  
342 Swedish population (Rizzo et al., 2009). Rizzo and coll. found significantly higher plasmatic  
343 sHLA-G level at delivery than during the 3rd trimester of pregnancy in allergic and non-  
344 allergic pregnant women. Moreover the progression of labour was associated with a  
345 continuous and significant increase in the sHLA-G level (Knafel et al., 2009). In Africa, the  
346 accessibility of health services is often difficult in rural area and most often pregnant women  
347 reach the maternity late (Thaddeus and Maine, 1994). In this context, our population study  
348 could contain a greater proportion of women with advanced progression of labor. The  
349 elevation of plasmatic sHLA-G could also be influence by others factors such as stress and  
350 hormonal changes induced during delivery.

351 In conclusion, our results argue for the first time for the potential involvement of HLA-G in the  
352 complex phenomenon of helminth-mediated immune tolerance during pregnancy.  
353 Investigation of this immune tolerance might help in furthering our understanding of the  
354 development of infant immune response.

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363

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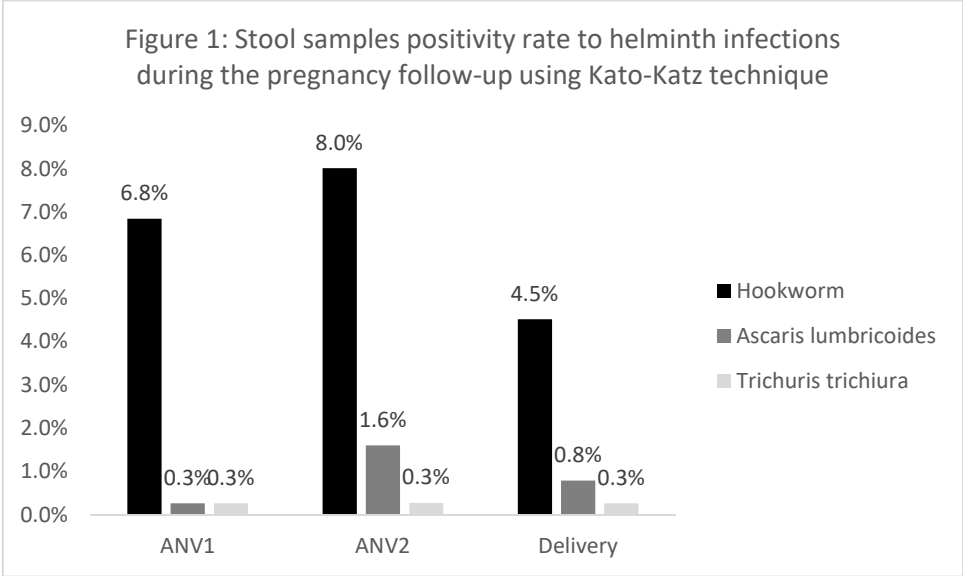
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529

Figure 1: Percentage of stool samples with helminth eggs during the pregnancy follow-up



A positive sample was defined as follow: stool sample with detection of helminth eggs by Kato-Katz method. Respectively 395, 387 and 355 stool samples were collected and examined at antenatal visit (AV)1, AV2 and at delivery.

Figure 2: Distribution of the adjusted mean of sHLA-G according to hookworm infection status of the women in the study

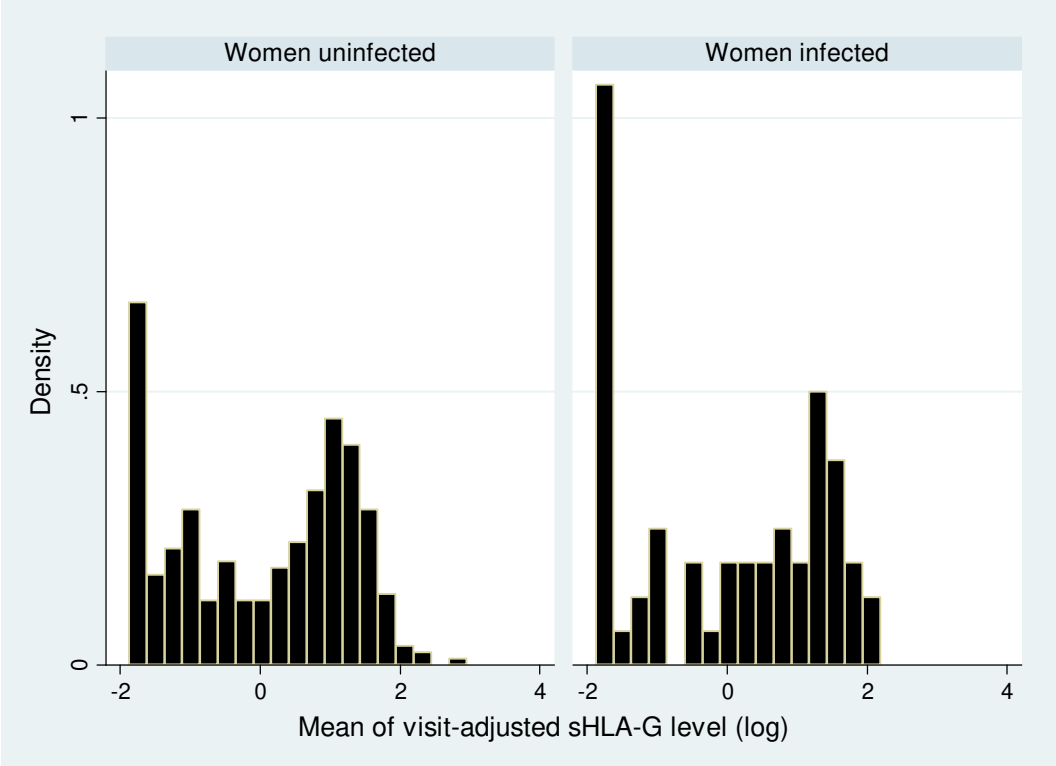
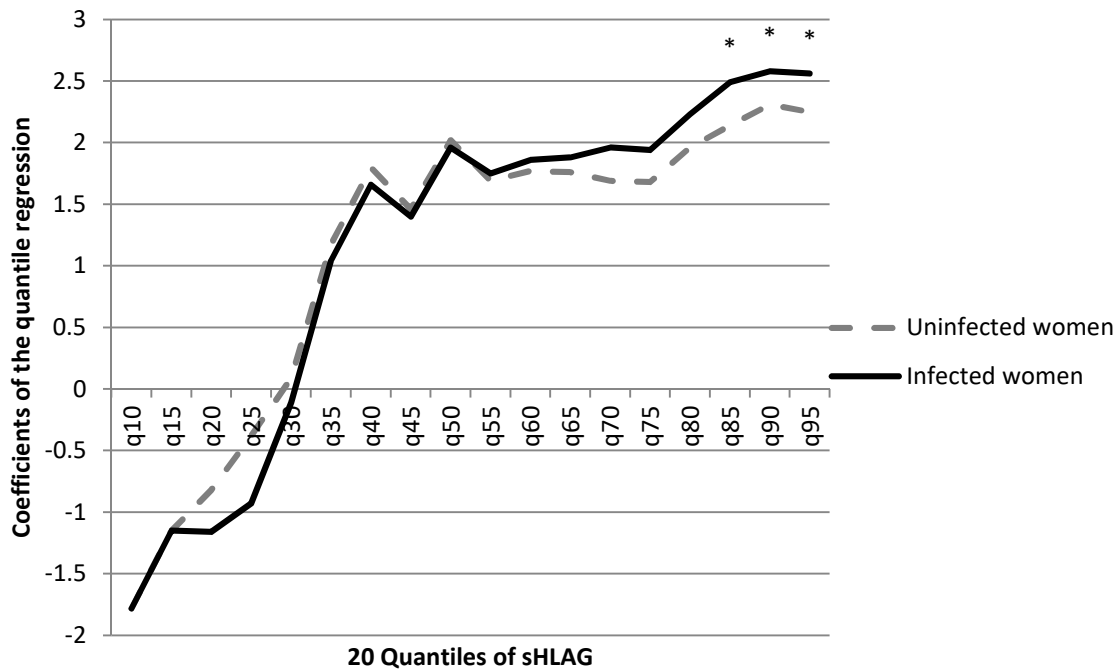


Figure 3: Effect of hookworm infection on the adjusted mean of sHLA-G controlled for inclusion center (Adjusted coefficients of quantile regression).



\*Quantiles with significant difference between coefficients of infected or uninfected women.

The curves show the regression coefficients on 20 quantiles of sHLA-G distribution during the follow-up, in women infected or non-infected with hookworms, controlled for the inclusion health facility. The graphic represents the effect of helminth infection on the quantiles of log HLA-G distribution. Significantly more hookworm-infected women were found above the Q80 percentile of sHLA-G distribution.

Table 1: Socio-demographic and economic characteristics of the participants

| <b>Variables</b>                 |                  | <b>N (%)</b> |
|----------------------------------|------------------|--------------|
| <b>Inclusion health facility</b> | Attogon          | 96 (24)      |
|                                  | Sékou            | 304 (76)     |
| <b>Marital status</b>            | Single           | 9 (2.3)      |
|                                  | Married          | 391 (97.7)   |
| <b>Occupation</b>                | Not working      | 223 (55.8)   |
|                                  | Working          | 177 (29.2)   |
| <b>Education</b>                 | Not educated     | 283 (70.8)   |
|                                  | Educated         | 117 (29.2)   |
| <b>Ethnicity</b>                 | Fon              | 83 (20.8)    |
|                                  | Aizo             | 278 (69.5)   |
|                                  | Others           | 39 (9.7)     |
| <b>Gravidity</b>                 | Multigravidae    | 337 (84.3)   |
|                                  | Primigravidae    | 63 (15.7)    |
| <b>Placental malaria</b>         | No               | 353 (88.3)   |
|                                  | Yes              | 43 (10.7)    |
|                                  | Unknown          | 4 (1)        |
| <b>IPTp regimen</b>              | SP               | 138 (34.5)   |
|                                  | MQ single dose   | 142 (35.5)   |
|                                  | MQ standard dose | 120 (30)     |
| <b>Household Latrines</b>        | No               | 231 (57.8)   |
|                                  | Yes              | 169 (42.2)   |
| <b>Electricity</b>               | No               | 262 (65.5)   |
|                                  | Yes              | 138 (34.5)   |
| <b>Refrigerator</b>              | No               | 384 (96)     |
|                                  | Yes              | 16 (4)       |
| <b>Television</b>                | No               | 267 (66.7)   |
|                                  | Yes              | 133 (33.2)   |
| <b>Motorbike</b>                 | No               | 90 (22.5)    |
|                                  | Yes              | 310 (77.5)   |

**Table 2:** Distribution of hookworm infections according to socio-demographic and economic characteristics of the study population

| <b>Variable</b>    |                  | <b>Hookworm infection*</b> |         |
|--------------------|------------------|----------------------------|---------|
|                    |                  | N(%)                       | p-value |
| Inclusion          |                  |                            |         |
| Health facility    | Attogon          | 8(8.3)                     | 0.02    |
|                    | Sékou            | 56(18.4)                   |         |
| Marital status     | Single           | 2(22.2)                    | 0.61    |
|                    | Married          | 62(15.9)                   |         |
| Occupation         | Not working      | 41(18.4)                   | 0.14    |
|                    | Working          | 23(13)                     |         |
| Education          | Not educated     | 48(17)                     | 0.41    |
|                    | Educated         | 16(13.7)                   |         |
| Ethnicity          | Fon              | 10(12.1)                   | 0.02    |
|                    | Aizo             | 53(19.1)                   |         |
|                    | Others           | 1                          |         |
| Gravidity          | Multigravidae    | 54(16)                     | 0.98    |
|                    | Primigravidae    | 10(15.9)                   |         |
| Placental malaria  | No               | 54(15.3)                   | 0.57    |
|                    | Yes              | 8(18.6)                    |         |
| IPTp regimen       | SP               | 17(12.3)                   | 0.30    |
|                    | MQ single dose   | 24(16.9)                   |         |
|                    | MQ standard dose | 23(19.2)                   |         |
| Household Latrines | No               | 49(21.2)                   | 0.001   |
|                    | Yes              | 15(8.9)                    |         |
| Wealth score       | <1               | 48(19.2)                   | 0.024   |
|                    | ≥1               | 16(10.7)                   |         |

\* At least one hookworm infection during the follow-up

**Table 3:** Effect of hookworm infection on 20 quantiles of sHLAG distribution during the follow-up. Crude coefficients and adjusted coefficients (controlled for inclusion center) of simultaneous quantile regression.

| Quantiles | Variable               | Crude $\beta^1$ | 95% CI       | P-value     | adjusted $\beta^2$ | 95% CI       | P-value     |
|-----------|------------------------|-----------------|--------------|-------------|--------------------|--------------|-------------|
| Q10       | Hookworm infection     | 0               | -0.01; 0.01  | 1           | 0                  | -0.07; 0.07  | 1           |
|           | Sekou health facility# | 0               | -0.19; 0.19  | 1           | 0                  | -0.21; 0.21  | 1           |
| Q15       | Hookworm infection     | -0.02           | -0.31; 0.26  | 0.87        | 0                  | -0.18; 0.18  | 1           |
|           | Sekou health facility  | -0.21           | -0.54; 0.12  | 0.21        | -0.21              | -0.53; 0.11  | 0.19        |
| Q20       | Hookworm infection     | -0.36           | -0.76; 0.05  | 0.08        | -0.34              | -0.73; 0.05  | 0.09        |
|           | Sekou health facility  | -0.27           | -0.85; 0.31  | 0.36        | -0.21              | -0.71; 0.29  | 0.42        |
| Q25       | Hookworm infection     | -0.55           | -1.22; 0.11  | 0.1         | -0.55              | -1.15; 0.05  | 0.07        |
|           | Sekou health facility  | -0.3            | -0.91; 0.31  | 0.34        | -0.29              | -0.79; 0.22  | 0.27        |
| Q30       | Hookworm infection     | -0.3            | -1.02; 0.42  | 0.41        | -0.2               | -0.99; 0.60  | 0.63        |
|           | Sekou health facility  | -0.4            | -1.14; 0.33  | 0.28        | -0.39              | -1.07; 0.29  | 0.26        |
| Q35       | Hookworm infection     | -0.18           | -1.02; 0.66  | 0.67        | -0.13              | -0.97; 0.71  | 0.76        |
|           | Sekou health facility  | -0.66           | -1.43; 0.11  | 0.09        | -0.69              | -1.42; 0.03  | 0.06        |
| Q40       | Hookworm infection     | -0.04           | -0.93; 0.85  | 0.93        | -0.14              | -1.10; 0.81  | 0.77        |
|           | Sekou health facility  | -0.72           | -1.54; 0.11  | 0.09        | -0.77              | -1.54; 0.01  | <b>0.05</b> |
| Q45       | Hookworm infection     | 0.004           | -0.87; 0.88  | 0.99        | -0.06              | -1.14; 1.01  | 0.91        |
|           | Sekou health facility  | -0.62           | -1.31; 0.08  | 0.08        | -0.57              | -1.30; 0.17  | 0.13        |
| Q50       | Hookworm infection     | -0.1            | -0.82; 0.62  | 0.78        | -0.06              | -1.03; 0.90  | 0.9         |
|           | Sekou health facility  | -0.65           | -1.28; -0.03 | <b>0.04</b> | -0.63              | -1.22; -0.04 | <b>0.04</b> |
| Q55       | Hookworm infection     | -0.09           | -0.75; 0.57  | 0.78        | 0.06               | -0.81; 0.93  | 0.89        |
|           | Sekou health facility  | -0.42           | -0.83; -0.01 | <b>0.05</b> | -0.42              | -0.87; 0.02  | 0.06        |
| Q60       | Hookworm infection     | -0.003          | -0.66; 0.47  | 0.74        | 0.09               | -0.56; 0.74  | 0.79        |
|           | Sekou health facility  | -0.37           | -0.68; -0.06 | <b>0.02</b> | -0.39              | -0.72; -0.06 | <b>0.02</b> |
| Q65       | Hookworm infection     | 0.2             | -0.49; 0.47  | 0.99        | 0.12               | -0.38; 0.62  | 0.64        |
|           | Sekou health facility  | -0.3            | -0.57; -0.03 | <b>0.03</b> | -0.32              | -0.62; -0.03 | <b>0.03</b> |
| Q70       | Hookworm infection     | 0.2             | -0.22; 0.61  | 0.35        | 0.27               | -0.13; 0.66  | 0.19        |
|           | Sekou health facility  | -0.25           | -0.47; -0.03 | <b>0.02</b> | -0.26              | -0.49; -0.03 | <b>0.03</b> |

|     |                       |       |                     |             |       |                     |             |
|-----|-----------------------|-------|---------------------|-------------|-------|---------------------|-------------|
| Q75 | Hookworm infection    | 0.19  | -0.18; 0.56         | 0.31        | 0.26  | -0.10; 0.62         | 0.15        |
|     | Sekou health facility | -0.17 | -0.42; 0.07         | 0.17        | -0.21 | -0.47; 0.04         | 0.10        |
| Q80 | Hookworm infection    | 0.22  | -0.10; 0.54         | 0.18        | 0.27  | -0.03; 0.58         | 0.08        |
|     | Sekou health facility | -0.23 | <b>-0.45; -0.01</b> | <b>0.04</b> | -0.27 | <b>-0.51; -0.03</b> | <b>0.03</b> |
| Q85 | Hookworm infection    | 0.24  | -0.02; 0.50         | 0.07        | 0.35  | <b>0.06; 0.64</b>   | <b>0.02</b> |
|     | Sekou health facility | -0.22 | <b>-0.43; -0.01</b> | <b>0.04</b> | -0.3  | <b>-0.52; -0.08</b> | <b>0.01</b> |
| Q90 | Hookworm infection    | 0.15  | -0.08; 0.38         | 0.21        | 0.27  | <b>0.01; 0.53</b>   | <b>0.04</b> |
|     | Sekou health facility | -0.23 | <b>-0.41; -0.06</b> | <b>0.01</b> | -0.31 | <b>-0.51; -0.11</b> | <b>0.01</b> |
| Q95 | Hookworm infection    | 0.17  | -0.11; 0.44         | 0.24        | 0.31  | <b>0.04; 0.58</b>   | <b>0.02</b> |
|     | Sekou health facility | -0.13 | -0.33; 0.07         | 0.21        | -0.23 | <b>-0.41; -0.06</b> | <b>0.01</b> |

<sup>1</sup>: From univariate quantile regression

<sup>2</sup>: From multivariate quantile regression

#: Reference: Attagon health facility