Placental malaria is associated with a TLR–Endothelin-3–oxidative damage response in human placenta tissues

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Abstract

Placental malaria, which is mainly caused by the sequestration of *Plasmodium falciparum*-infected erythrocytes in the placenta, is an important driver of poor pregnancy outcomes, including fetal growth restriction, preterm birth, and stillbirth. However, the mechanisms underlying its adverse outcomes are unclear. Mouse models have previously shown that placental malaria triggers a proinflammatory response in the placenta, which is accompanied by a fetal Toll-like receptor (TLR)4-mediated innate immune response associated with improved fetal outcomes. Here, we used hematoxylin and eosin staining to identify placental malaria positive and negative samples in our biobank of placentas donated by women living in a malaria-endemic region of Kenya and assessed the impact of placental malaria on the expression of TLRs, Endothelins, and oxidative damage. RT-qPCR analysis revealed that placental malaria was associated with an upregulation of TLR4, TLR7, and Endothelin-3. Moreover, immunohistochemistry showed that placental malaria was associated with elevated expression levels of the oxidative DNA damage marker, 8-hydroxy-2'-deoxyguanosine, while RT-qPCR revealed that this was accompanied by an upregulation of p21, an inhibitor of cell cycle progression and marker of cellular response to DNA damage. These findings allude to a novel mechanism of placental malaria pathogenesis driven by a TLR–Endothelin-3–oxidative DNA damage signaling axis.

Keywords: Placental malaria, malaria in pregnancy, poor pregnancy outcomes, innate immunity, *Plasmodium falciparum*

1. Introduction

According to the World Health Organization, globally, there were about 249 million malaria cases and 608,000 malaria-associated deaths in 2022, with sub-Saharan Africa accounting for most of the cases and deaths [1]. Pregnant women have a higher susceptibility to malaria infection [2] and it is estimated that in 2022, there were about 12.7 million cases of malaria in pregnancy in sub-Saharan Africa [1]. Malaria in pregnancy is associated with several adverse outcomes in the mother, fetus, and neonate [3]. For the fetus, malaria in pregnancy severely worsens pregnancy outcomes and frequently leads to fetal growth restriction (including low birthweight, small for gestational age, and intrauterine growth restriction) and may result in preterm birth and stillbirth [3–5].

The adverse effects of malaria in pregnancy on the fetus are attributable to malaria infection of the placenta [1], leading to placental malaria. Placental malaria is characterized by the sequestration of *Plasmodium*-infected erythrocytes in placental intervillous spaces. This phenomenon is most frequently associated with *Plasmodium falciparum* (*P. falciparum*), the species associated with the most severe form of malaria [6]. The sequestration of *P. falciparum*-infected erythrocytes in the placenta is mediated by the interaction between variant surface chondroitin surface antigen 2, a *Plasmodium falciparum* protein expressed on the surface of infected erythrocytes [7], and chondroitin sulfate A on the surface of the syncytiotrophoblast [7], the placental epithelial cell layer that contacts maternal blood [8].

The adverse impacts of placental malaria on fetal well-being most likely result from the negative effects of placental malaria on placental health and function, since the vertical transmission of malaria to the fetus is rare [9]. Indeed, placental malaria is reported to induce placental inflammation [10,11] and placental histological changes [12], which may contribute to placental insufficiency and poor pregnancy outcomes. However, the mechanisms underlying placental

71 malaria-driven placental pathobiology are not fully understood at the cellular and cell signaling
72 levels.

73 Innate immune factors, such as Toll-like receptor (TLR)4, 7, and 9, which respond to infection by
74 recognizing invading pathogens for clearance, are reported to respond to malaria infection [13],
75 although their role in placental malaria is unclear. Although several studies have investigated
76 maternal responses to placental malaria, few have studied how the fetus responds to parasite
77 sequestration in the placenta. Nonetheless, a mouse model revealed that placental malaria
78 triggers a TLR4-mediated innate immune reaction that adversely affects fetal outcomes, which is
79 countered by a fetal innate immune reaction that led to better pregnancy outcomes [14]. This
80 suggests the presence of TLR-mediated innate immune responses to placental malaria, although
81 this has not been reported in the context of human placental malaria. Here, considering that
82 mouse data show that TLR4 modulates endothelin-1 expression [15], malaria is inflammatory and
83 oxidative [16], oxidative DNA damage upregulates TLR4 [17], and TLR signaling is thought to
84 promote DNA repair [18], we used biobank placenta samples donated by women living in a
85 malaria-endemic region of Kenya to examine the hypothesis that human placental malaria triggers
86 a TLR–Endothelin–oxidative damage signaling response.

87 **2. Materials and methods**

88 **2.1 The biobank and study participants**

89 The study used biobank placenta samples donated by residents of Bungoma County, a malaria-
90 endemic region of Western Kenya. The biobank was established by a previous prospective parent
91 study. All placenta donors were aged ≥18 years and gave written informed consent to participate
92 in the parent study. Based on questionnaire responses, participants with a known record of
93 sexually transmitted disease infection during pregnancy, those with pregnancy-associated
94 noncommunicable diseases (preeclampsia and gestational diabetes) during the current
95 pregnancy, and those with twin pregnancies were excluded from our analyses. During participant

96 recruitment and sample collection, participants were recorded as having malaria in pregnancy if
97 they had at least one episode of hospital-diagnosed malaria during pregnancy. Maternal malaria
98 status was diagnosed using a rapid diagnostic test (Malaria Ag P.f, SD Biosensor). All data
99 underlying the databank were deidentified. The characteristics of the biobank's placenta donors
100 and samples are summarized in Table 1. Equal numbers of male and female placentas were
101 analyzed. Demographic data (Table 1), including maternal age, history of malaria during
102 pregnancy, and gravidity were collected using questionnaires, whereas pregnancy-associated
103 data, including birthweight and placental weight, were recorded after birth. All participants gave
104 written informed consent before joining the study and agreed to the collection and use of their
105 placenta samples in the study.

106 **2.2 Histological analysis**

107 Formalin-fixed placenta tissues were embedded in paraffin blocks as previously described [19]
108 using an automated tissue embedding system (MediMeas). H&E analysis was used to confirm
109 placental malaria presence, which is indicated by the presence of infected erythrocytes in the
110 placenta. Briefly, formalin-fixed paraffin-embedded samples were sectioned onto charged
111 microscope slides (Dako, Cat No. K8020) at a 5-μm thickness, dried at 37 °C overnight on a slide
112 warmer, dewaxed in xylene (Finar Chemicals, Cat No. 21940LC250), rehydrated by dipping
113 across an alcohol gradient of absolute, 95%, 70%, and 50% ethanol (Scharlau, Cat No.
114 ET00052500), and then in distilled water. They were then submerged in hematoxylin (Loba
115 Chemie, Cat No. 04023) for seven minutes, rinsed with running water, and then destained through
116 10 dips in acid alcohol (1% hydrochloric acid in 70% ethanol). Next, they were submerged in eosin
117 (Griffchem, Cat No. 45380) for 45 seconds followed by dehydration in 95% ethanol and absolute
118 ethanol (five minutes each) and then cleared in xylene baths (10 minutes each) before being
119 cover-slipped using dibutylphthalate polystyrene xylene mountant (Finar Chemicals, Cat No.
120 10525LM250). The slides were then examined under a microscope (Richter Optica UX1, M2

121 Scientifics), followed by imaging in ≥10 fields of view per slide at a 40X magnification using a
122 Moticam microscope camera (Motic Scientific). Placental malaria was then diagnosed as
123 described before (Odongo et al., 2016) based on the presence of infected erythrocytes. Placental
124 malaria-associated histopathological features were assessed by counting the number of syncytial
125 knots as described previously [20] and measuring the fibrin-occupied placental areas using
126 imageJ. These analyses were done in at least 10 fields of view per sample. The levels of placental
127 malaria burden in the placental malaria-positive samples were determined by counting the
128 number of identified infected erythrocytes in at least 10 fields of view per slide imaged at a
129 magnification of 40X.

130 **2.4 RNA extraction and reverse transcription quantitative PCR (RT-qPCR)**

131 Total RNA was extracted from placenta tissue using a HigherPurity™ Tissue Total RNA
132 Purification kit as per the manufacturer's guidelines (Canvax, cat No. AN0152) and quantified
133 using a NanoDrop Microvolume Spectrophotometer (ThermoFisher Scientific) following the
134 manufacturer's instructions. For each sample, 500 ng of RNA were retrotranscribed into cDNA
135 using a LunaScript™ RT SuperMix cDNA Synthesis Kit (NEB, Cat. No. E3010L) using the
136 manufacturer's protocol. RT-qPCR analysis was done on a QuantStudio™ 5 Real-Time PCR
137 System in a final volume of 20 µl containing 10 µl of GoTaq qPCR Master Mix (Promega, Cat No.
138 PRA6001), 2 µl of the forward plus reverse primers (final primer concentration: 500 nM), 3 µl of
139 nuclease free water (Promega, Cat No. P119E), and 5 µl of cDNA using the following program:
140 50 °C for two minutes, 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds and
141 60°C for 30 seconds. Relative gene expression was determined using the $2^{-\Delta\Delta ct}$ method [21], using
142 β-actin as the reference gene. Primers were purchased from Macrogen and primer sequences
143 are provided in Table 3.

144 **2.5 *P. falciparum* detection PCR**

145 The presence of *P. falciparum* in placenta samples was evaluated using One Taq® Quick-Load®
146 2X Master Mix with Standard Buffer (NEB, Cat No. M0486L) and the following thermocycler
147 program: Initial denaturation at 95 °C for five minutes, followed by 35 cycles of denaturation at 95
148 °C for 30 seconds, annealing at 55 °C for 60 seconds, and extension at 72 °C for 75 seconds,
149 and then a final extension at 72 °C for five minutes. Primer sequences are shown in Table 3. The
150 PCR product was subjected to agarose (Sigma–Aldrich, Cat No. A9539) gel electrophoresis using
151 1X tris–borate–EDTA buffer alongside a 100 base pair ladder using SafeView™ Classic (Applied
152 Biological Materials, Cat No. G108) nucleic acid stain and Gel Loading Dye, Purple (6X) (NEB,
153 Cat No. B7024S). The bands were developed and imaged using a UVITEC Gel Documentation
154 System (Cleaver Scientific).

155 **2.6 Immunohistochemistry**

156 The sections were deparaffinized by warming at 55 °C for 15 minutes followed by dipping in three
157 xylene baths, about 10 dips each. They were then rehydrated and subjected to heat-induced
158 epitope retrieval by boiling for 30 minutes in Citrate Buffer, pH 6.0 (Sigma–Aldrich, cat. No.
159 C9999). They were then cooled to room temperature, rinsed with distilled water for five minutes
160 and then blocked with 0.3% Triton-X in 1X phosphate-buffered saline (PBST). Next, they were
161 blocked with 10% normal donkey serum (Abcam, cat. No. ab7475) in PBST for two hours followed
162 by overnight incubation (4 °C) with anti-DNA/RNA damage antibody [15A3] (Abcam, cat. No.
163 ab62623) at 1:2500 in blocking solution. Sections were then washed thrice (10 minutes each)
164 using PBST and then incubated at room temperature for two hours with horseradish peroxidase-
165 conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, cat. No. 115-035-
166 003) at 1:5000 in blocking solution. The sections were then washed thrice (10 minutes each) using
167 PBST followed by signal development using an ImmPACT® DAB Substrate Kit (Vector, cat. No.
168 SK-4105) as per the manufacturer's protocol. They were then dehydrated using 95%, 95%, 100%,
169 and 100% ethanol (five minutes each), cleared by dipping in three xylene baths and then cover-

170 slipped using a xylene-based mountant and allowed to dry. They were then examined under a
171 light microscope and imaged at a magnification of 40X.

172 **2.7 Data analyses**

173 Statistical analyses were done using GraphPad Prism version 9. Data are presented as
174 percentages, raw values, or mean ± standard deviation. Differences between two groups were
175 compared using a t-test. Correlation analyses were done using nonparametric Spearman
176 correlation analysis. For each placenta, the placental malaria burden in placentas was indicated
177 by the total number of infected erythrocytes in the placenta section. The birthweight-to-placenta
178 weight ratio was obtained by dividing the birthweight (grams) with the corresponding placenta's
179 weight (grams). The correlation between placental malaria status and birthweight, placental
180 weight, and birthweight-to-placental weight ratio was assessed using GraphPad prism to examine
181 the impact of placental malaria on fetal outcomes. $P < 0.05$ indicates statistically significant
182 differences.

183 **3. Results**

184 **3.1 Main characteristics of the placenta donors and donated placentas**

185 All placenta donors were ≥18-years-old and had received intermittent presumptive treatment with
186 sulfadoxine pyrimethamine. The mean age, gravidity, birthweight, placental weight, and
187 birthweight-to-placental weight ratio of the cohort of placenta donors were 24.7 years, 2.69,
188 3077.64 g, 470.04 g, and 6.51, respectively (Table 1). Grouping the placenta donors into those
189 with a known history of malaria in pregnancy (MiP) and those without (NoMiP), revealed that in
190 the MiP vs NoMiP groups, maternal age and gravidity were not significantly different (mean age:
191 24.4 [range: 18–30] vs 25 [range: 18–40] years, $P = 0.45$; mean gravidity: 2.5 [range: 1–7] vs 2.9
192 [range: 1–7), $P = 0.12$). However, in the MiP vs NoMiP groups, birthweight (mean: 2870.5 [range:
193 1600–5000] vs 3272.3 [range: 2000–4500) g), placental weight (mean: 464.3 [range: 280.4–675)
194 vs 492.9 [range: 342.3–715] g, and birthweight-to-placental weight ratio (mean: 6.27 [range: 3.2–

195 10.2] vs 6.73 [range: 4.18–10], were significantly lower in the MiP group ($P < 0.0001$, = 0.009,
196 and = 0.03, respectively). H&E analysis revealed that 92 placentas (51.4%) were placental
197 malaria-positive (had infected erythrocytes), 58 (32.4%) were placental malaria-negative (no
198 infected erythrocytes observed), 29 (16.2%) had past malaria infection (hemozoin present in the
199 absence of infected erythrocytes [12]), 92 (51.4%) belonged to male babies, and eight (4.5%)
200 were preterm (Table 2). All downstream analyses were done on placenta samples that were
201 confirmed to be placental malaria-positive or placental malaria-negative using H&E staining.

202 **3.2 Placental malaria correlates negatively with birthweight and birthweight-to-placenta**
203 **ratio**

204 Representative images of placental malaria-negative and placental malaria-positive samples are
205 shown in Figure 1A–B, and the presence of *P. falciparum* in placental malaria-positive tissues
206 was confirmed using PCR (Figure 1C). Analysis of the data underlying the placental biobank
207 revealed that when compared with the placental malaria-negative group, birthweight was
208 significantly lower in the placental malaria-positive group, which had more low birthweight cases
209 (Figure 1A, $P = 0.03$, low birthweight: <2500 g), but placental weight did not differ between the
210 two groups (Figure 2B, $P = 0.8$). However, the birthweight-to-placental weight ratio was lower in
211 the placental malaria-positive group, although the difference did not reach statistical significance
212 (Figure 1F, $P = 0.08$). Next, we used the H&E images to determine the proportion (%) of infected
213 erythrocytes (IEs) in each placenta sample, and then used the obtained values to assess the
214 correlation between the placental malaria burden and birthweight, placental weight, and the
215 birthweight-to-placental weight ratio, i.e., the fetal weight obtained per gram of the placenta, which
216 is an indicator of placental efficiency, with higher birthweight-to-placental weight ratios indicating
217 greater placental efficiency [22]. This analysis revealed negative correlation between IEs
218 (proportion of infected erythrocytes [%]) and birthweight (correlation coefficient [rs]: -0.22, $P <$
219 0.005, 95% confidence interval [CI]: -0.359 – -0.071), and IEs (%) and birthweight-to-placental

220 weight ratio (rs: -0.20, P = 0.007, 95% CI: -0.338 – -0.048). Expectedly, placenta weight had a
221 positive correlation with birthweight (rs: 0.29, P < 0.001, 95% CI: 0.141 – 0.419) and a negative
222 correlation with birthweight-to-placental weight ratio (rs: -0.42, P < 0.001, 95% CI: -0.539 – -
223 0.290). However, placental malaria burden did not exhibit correlation with placenta weight (rs:
224 0.01, P = 0.94, 95% CI: -0.156 – 0.146). Taken together, these findings indicate that placental
225 malaria impairs placenta function, leading to low birthweight via reduced placenta efficiency as
226 indicated by the negative correlation between placental malaria burden and the birthweight-to-
227 placental weight ratios of placental malaria-exposed neonates.

228 **3.3 Placental malaria markedly alters placental histological features**

229 Next, we assessed the impact of placental malaria on syncytial knotting and fibrin deposition in
230 our placenta samples. This analysis revealed that when compared with placental malaria-negative
231 samples (A), placental malaria-positive samples had more syncytial knots (B, yellow arrowhead)
232 and greater fibrin-occupied placental area (C, FD [fibrin deposit] in the broken line-demarcated
233 area). Quantification analyses revealed that when compared with placental malaria-negative
234 samples, the levels of these histological features were significantly higher in placental malaria-
235 positive samples (D, SK [syncytial knots], P = 0.047 and E, fibrin area, P < 0.0005). These
236 observations indicate that in our study cohort, placental malaria may have adversely affected fetal
237 outcomes by at least in part, altering normal placental histological features.

238 **3.4 Placental malaria is associated with an upregulation of TLR4, TLR7, and Endothelin 3**

239 We then sought to determine if placental malaria altered the expression of TLRs. To this end, we
240 focused on TLR4, TLR7, and TLR9, which have been associated with response to malaria
241 infection in mice [23,24], and with mouse placental malaria in the case of TLR4 [25], although this
242 has not been reported in human placental malaria. To evaluate the effect of placental malaria on
243 these innate immune system receptors, we assessed their expression levels using RT-qPCR. The
244 analysis revealed that when compared with placental malaria-negative controls, placental

245 malaria-positive samples expressed significantly higher levels of TLR4 and TLR7, but not TLR9
246 (Figure 3A–C, P = 0.002, 0.03, and 0.59, respectively). This is consistent with mouse data
247 showing that placental malaria upregulates TLR4-mediated expression of endothelin-1 [15]. We
248 therefore wondered if human placental malaria alters the expression of Endothelin genes. RT-
249 qPCR analysis of Endothelin-1 and -3 gene expression revealed that only Endothelin-3 was
250 detectable in our placenta samples and that when compared with placental malaria-negative
251 placentas, placental malaria-positive samples had significantly higher Endothelin-3 levels (Figure
252 3D, P = 0.004), indicating the presence of a TLR–Endothelin signaling axis in response to human
253 placental malaria.

254 **3.5 Placental malaria is associated with high oxidative DNA damage**

255 Since TLR4 was upregulated in placental malaria-positive placenta samples, we wondered
256 whether placental malaria-driven TLR4 activation is associated with a dysregulation of other
257 signaling processes that may underlie or contribute to placental malaria-mediated placental
258 pathobiology. Because malaria is known to be strongly inflammatory and oxidative, which may
259 drive host tissue damage [16], and because oxidative DNA damage is associated with TLR4
260 upregulation [17] and that TLR signaling is thought to promote DNA repair [18], we wondered if
261 the TLR4 upregulation in the placental malaria-positive samples might be associated with
262 placental oxidative DNA damage. To assess this possibility, we used immunohistochemistry to
263 assess the levels of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative DNA stress [26]. This
264 analysis revealed that when compared with placental malaria-negative samples, placental
265 malaria-positive samples express markedly higher levels of 8-hydroxy-2'-deoxyguanosine (Figure
266 4A). Staining the same samples with the secondary antibody only (without the primary antibody)
267 confirmed signal specificity (Figure 4B). To further assess the effect of placental malaria on
268 oxidative stress, we used RT-qPCR to examine the level of p21, a mediator of cell cycle arrest
269 and indicator of cellular response to DNA damage [27]. This revealed that when compared with

270 placental malaria-negative samples, placental malaria-positive samples had significantly higher
271 levels of p21 (Figure 4C, P = 0.02). Taken together, these data indicate that placental malaria
272 triggers markedly high levels of placental oxidative DNA stress, placenta tissue damage, and
273 cellular response to DNA stress, which may contribute to placental malaria pathobiology, and that
274 in response, TLR signaling may at least in part, be upregulated to counter these adverse effects
275 through the promotion of DNA repair.

276 **4. Discussion**

277 Malaria in pregnancy often results in placental malaria, where erythrocytes that are infected with
278 *P. falciparum*, the parasite that most frequently causes placental malaria, sequester in placental
279 intervillous spaces [7]. Placental malaria may cause various adverse fetal outcomes, including
280 stillbirth, preterm birth, and fetal growth restriction [3–5], and because *P. falciparum* rarely
281 undergoes vertical transmission [9], these effects are likely caused by placental malaria-driven
282 pathobiological effects in the placenta, which may impair placental function. However, although
283 studies have implicated effects like inflammation and histological changes in placental malaria
284 pathogenesis, the mechanisms underlying the adverse effects of human placental malaria on the
285 placenta are unclear. Moreover, because many malaria in pregnancy cases in malaria-endemic
286 regions are asymptomatic [28,29] and the placenta is inaccessible during pregnancy, there are
287 no ways of detecting and intervening against placental malaria during pregnancy. Thus, there is
288 an urgent need to better understand the placental pathobiology of placental malaria to guide the
289 development of effective diagnostic and therapeutic tools.

290 Mouse models indicate that placental malaria triggers innate immune responses (mainly via
291 TLR4) that are associated with poor fetal outcomes and that TLR4-mediated fetal responses to
292 placental malaria cause improved outcomes [14]. However, the effect of human placental malaria
293 on TLR-mediated immunity in the placenta has not been examined. In this study, we leveraged
294 our well-characterized biobank of placenta samples from a malaria endemic region of Kenya

295 (Table 1) and found that in our study cohort, placental malaria burden had a significant negative
296 correlation with birthweight and birthweight-to-placental weight ratio, that it was associated with
297 significantly higher placental histological lesions, higher levels of TLR4 and Endothelin-3
298 expression, and enhanced oxidative DNA damage when compared with samples without
299 placental malaria.

300 Consistent with previous findings implicating placental malaria in fetal growth restriction [30], we
301 observed that in our study cohort, relative to the placental malaria-negative cases, placental
302 malaria was associated with low birthweight. Moreover, we observed that placental malaria was
303 associated with lower birthweight-to-placental weight ratios, an indicator of placental efficiency in
304 which higher ratios indicate higher nutrient transfer for every gram of the placenta and vice versa
305 [22], but not with lower placental weight (Figure 1D–F), an observation that to our knowledge, has
306 not been previously reported in human placental malaria. Interestingly, our analyses also indicate
307 that the placental malaria burden (percentage of infected erythrocytes in a sample's intervillous
308 spaces) correlates negatively with birthweight but not with placental weight. Taken together, these
309 observations indicate that placental malaria contributes to fetal growth restriction primarily by
310 impairing placental function, and not via placental growth inhibition, although the precise
311 mechanisms remain unclear. This possibility is crucial considering that women in malaria-endemic
312 regions may experience multiple malaria reinfections throughout pregnancy. Therefore, further
313 studies, such as using *in vitro* and organoid systems, are needed to comprehensively investigate
314 this possibility.

315 Our analyses revealed that, placental malaria-positive samples had markedly higher levels of
316 fibrin deposition and syncytial knotting than placental malaria-negative samples, which is in line
317 with earlier findings [31]. These changes, which indicate placental injury and have been
318 associated with placental malperfusion and poor fetal outcomes, including fetal growth restriction
319 [32,33], may contribute to the low birthweight observed in our placental malaria cohort. However,

320 studies are needed to establish the mechanisms by which placental malaria alters placental
321 histological features, how these changes correlate with fetal outcomes and postnatal wellbeing,
322 and whether they can predict fetal wellbeing in postnatal life.

323 TLRs are key innate immunity factors that sense host invasion by pathogens and activate host
324 immune defenses [34]. Mouse models of malaria indicate that TLR4, TLR7, and TLR9 are
325 involved in detecting and responding to malaria infection [23,24]. Moreover, mouse models
326 indicate that at the fetal–maternal interface, placental malaria activates TLR4-mediated immune
327 responses that drive poor fetal outcomes, and that fetal TLR4-mediated counterresponses
328 improve pregnancy outcomes [14,25,35]. However, this observation has not been previously
329 made in human placental malaria. Here, we observed that the expression levels of TLR4 and
330 TLR7, but not TLR9, were significantly upregulated in placental malaria-positive samples,
331 indicating that placental infection triggers an innate immune reaction and that it is mainly driven
332 by TLR4 and TLR7, although the status of other TLRs during placental malaria warrants
333 investigation. Considering that TLRs are important drivers of inflammation [36], which is
334 implicated in placental malaria pathogenesis [37], taken together with the observed changes in
335 placental histological features, our findings indicate for the first time, that TLR-mediated
336 responses to placental malaria may contribute to local placental inflammation, which may underlie
337 the observed placental malaria-associated low birthweight and placental weight, although the
338 precise mechanisms remain unclear. Mouse data show that placental malaria-driven TLR4
339 expression drives placental endothelin-1 expression [15], and for the first time, our findings show
340 that placental malaria is also associated with the upregulation of Endothelin-3. The Endothelin
341 ligands 1, 2, and 3 are a family of vasoactive factors that influence a range of cellular processes,
342 such as vascular remodeling and angiogenesis [38]. Moreover, Endothelin-3 is reported to have
343 anti-inflammatory effects [39,40], suggesting that its upregulation in the context of placental
344 malaria-mediated TLR4 upregulation is a mechanism of countering TLR4-dependent placenta

345 inflammation. Collectively, these observations indicate that human placental malaria may activate
346 a TLR4–Endothelin-3 signaling axis, but further studies are needed to test this hypothesis and to
347 determine its implications in placental malaria pathobiology and fetal outcomes.

348 Based on reports that malaria is strongly oxidative [16], oxidative stress causes tissue damage
349 [41], oxidative DNA damage upregulates TLR4 [17], and that the TLR pathway might promote
350 DNA repair [18], we reasoned that our observation of TLR4 and TLR7 upregulation in placental
351 malaria-positive samples might be accompanied by placental oxidative DNA damage. This
352 hypothesis was confirmed by our immunohistochemistry data, which showed that 8-hydroxy-2'-
353 deoxyguanosine, a marker of oxidative DNA stress [26], was markedly upregulated in placental
354 malaria samples. Moreover, gene expression analysis revealed that these events were
355 accompanied by a significant upregulation of p21, a cell cycle inhibitor and marker of cellular
356 response to DNA damage [27]. These observations align with previous findings that in a mouse
357 model, placental malaria is associated with placental oxidative damage [42]. Furthermore, p21
358 upregulation in the placenta may arrest the cell cycle to allow for oxidative damage resolution,
359 which may contribute to the low placental weight observed in our cohort, but this possibility
360 requires further investigation. Together, our data suggest the presence of a previously unknown
361 TLR–Endothelin–oxidative damage axis in human placental malaria.

362 Finally, our data also show that in the placental malaria-negative and placental malaria-positive
363 groups, the analyzed parameters varied across samples, which may reflect inter-individual
364 biological variability [43]. In the future, our observations will be validated experimentally, such as
365 by challenging cultured primary human trophoblasts with *P. falciparum* for detailed mechanistic
366 interrogation.

367 **5. Conclusion**

368 Despite its heavy burden and adverse effects on maternal and fetal outcomes, the mechanisms
369 underlying the placental pathobiology of placental malaria are unclear. Considering that malaria

370 is rarely transmitted to the fetus [9], the adverse fetal outcomes of malaria in pregnancy are mainly
371 driven by events that disrupt placenta physiology and function. Importantly, because of placental
372 inaccessibility, placental malaria can only be confirmed via postnatal placental histopathology.
373 These challenges highlight the urgent need to better understand the mechanisms underlying the
374 placental pathobiology of placental malaria, which may inform the development of sensitive tools
375 for diagnosing placental malaria during pregnancy as well as effective therapeutic interventions.
376 Our findings that placental malaria may drive TLR-mediated responses in the placenta raise the
377 possibility that modulating innate responses to placental malaria may improve fetal outcomes, as
378 we previously discussed [13]. Moreover, our identification of an axis involving TLRs, Endothelins,
379 and oxidative DNA damage during placental malaria (Figure 5) highlights processes with the
380 potential for intervention against human placental malaria. However, further studies, such as using
381 primary human trophoblasts, human placental organoids, or human placental *ex vivo* systems are
382 needed to validate our observations. Such approaches can investigate the mechanisms of
383 placental malaria pathobiology more rigorously than can be done using term placentas.

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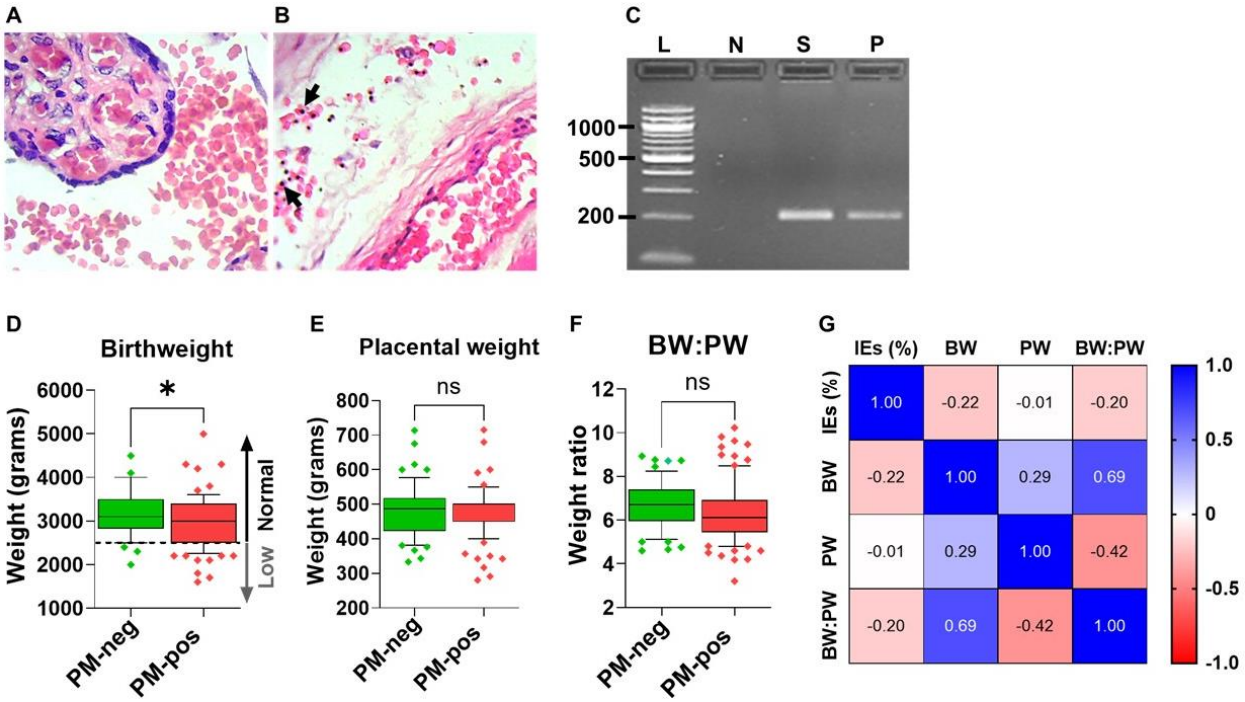
Conflict of interest

The authors declare no conflicts of interest.

Ethics statement

This study was approved by Mount Kenya University's ethics review committee (approval number 1314).

415 **Figures**



416

417 **Figure 1.** (A–B) Representative hematoxylin and eosin images of placental malaria (PM)-negative
418 (A) and PM-positive samples (B). When compared with a PM-negative sample, the positive
419 sample has malaria-infected erythrocytes (black arrowheads) in the placental intervillous space.
420 (C) PCR confirmed the presence *of P. falciparum* in the PM-positive sample in B. L: 100 base pair
421 ladder, N: PM-negative sample, S: PM-positive sample in B (histology), P: positive control (*P.*
422 *falciparum* strain 3D7 genomic DNA). Expected PCR band size: 205 base pairs. (D–F) PM was
423 associated with lower birthweight (BW) (D, P = 0.03) and lower birthweight-to-placental weight
424 (BW:PW) ratio, although the difference did not reach statistical significance (F, P = 0.08), but not
425 with lower placental weight (PW) (E, P = 0.8). In D–F, whiskers are drawn from the 10th to 90th
426 percentile. (G) A correlation matrix shows that the proportion of infected erythrocytes, IEs (%), in
427 the placenta correlated negatively with BW (P < 0.005) and the BW:PW ratio (P = 0.007), but it
428 did not correlate with PW (P = 0.94). PW correlated positively with BW and negatively with the
429 BW:PW ratio (both P < 0.001).

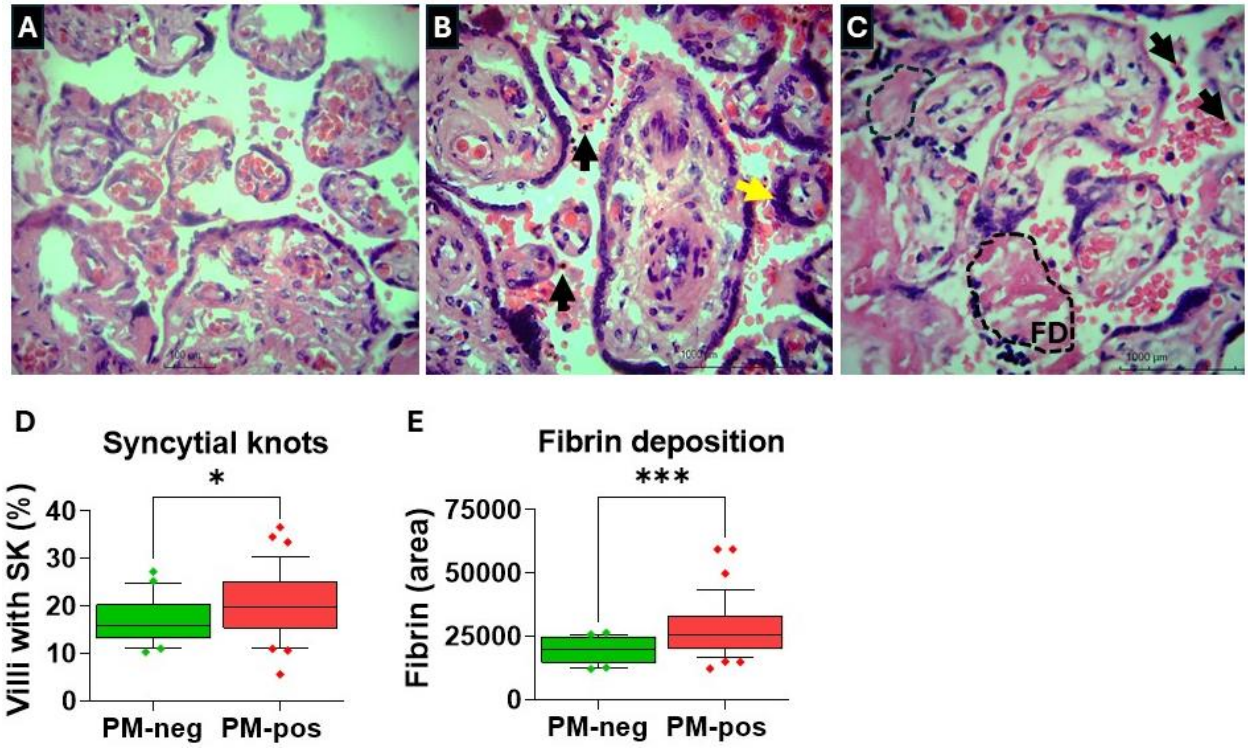


Figure 2. (A–C) In the samples from the biobank underlying our study, when compared with placental malaria (PM)-negative samples (A), PM was associated with significantly higher rates of syncytial knots (B, yellow arrowhead) and placental fibrin deposits (C; FD, marked with broken line). Black arrowheads indicate infected erythrocytes. (D–E) Quantification revealed that the levels of syncytial knots (SK [D], n = 21 and 38 for PM-neg and PM-pos, respectively; $P = 0.047$) and the area of placenta intervillous space containing fibrin (E, n = 26 and 38 for PM-neg and PM-pos, respectively, $P < 0.0005$) were significantly higher in the PM-positive (PM-pos) samples than in the PM-negative (PM-neg) samples. Whiskers are drawn from the 10th to 90th percentile.

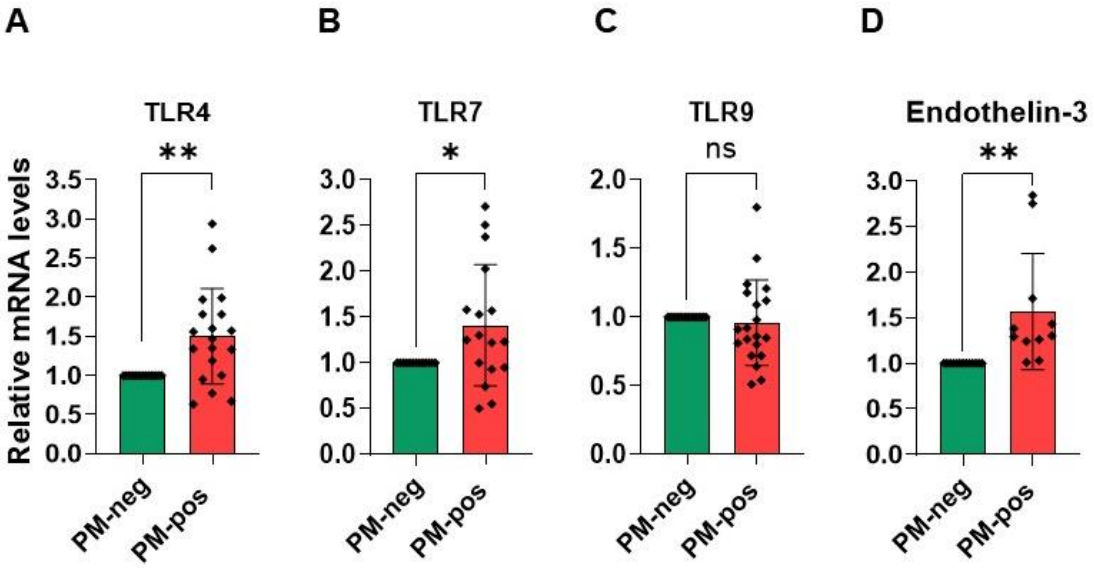
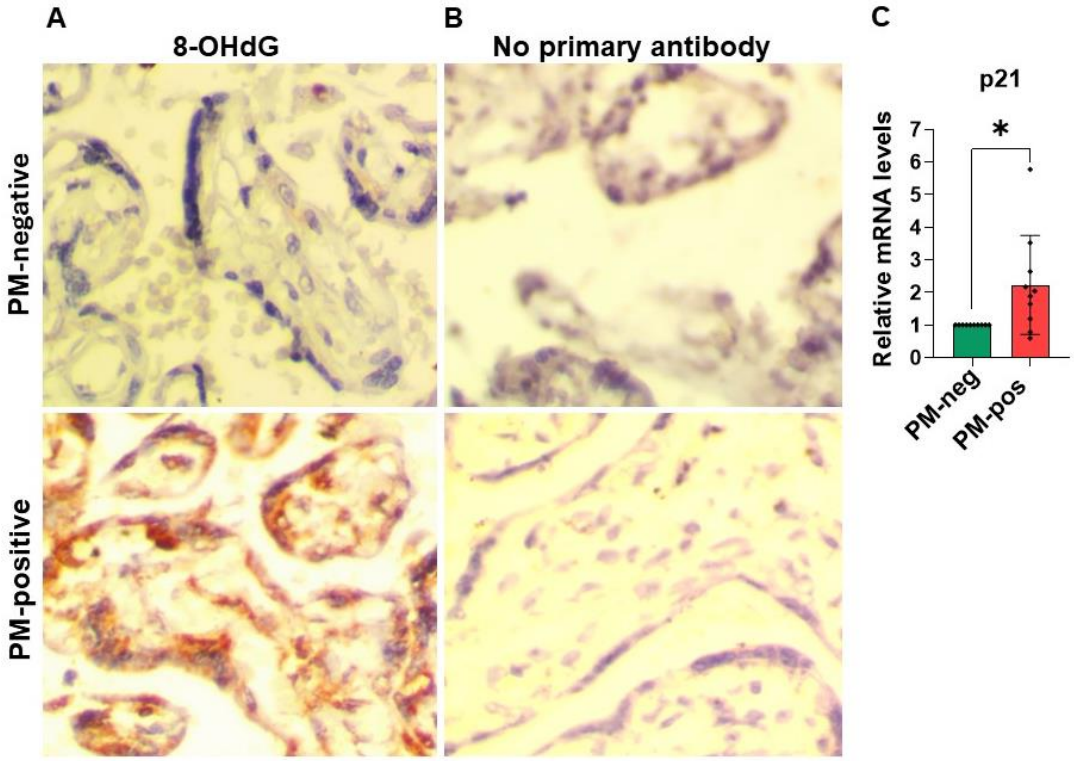


Figure 3. Placental malaria is associated with the upregulation of TLR4, TLR7, and Endothelin-3. (A–C) When compared with placental malaria (PM)-negative samples (PM-neg), PM-positive (PM-pos) samples expressed significantly higher levels of TLR4 and TLR7, but not TLR9 (P = 0.002, 0.03, and 0.59, respectively). (D) PM-positive samples also expressed higher levels of Endothelin-3 (P = 0.004).



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449 **Figure 4.** Analysis of oxidative DNA damage in placental malaria (PM)-negative vs PM-positive
450 samples. (A–B) Immunohistochemistry revealed that when compared with PM-negative samples,
451 PM-positive tissues had markedly higher levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), an
452 indicator of oxidative damage. Staining the same samples with the secondary antibody only (B)
453 confirmed signal specificity for this marker. (C) RT-qPCR showed that PM-positive samples
454 express significantly higher levels of p21 (P = 0.02).

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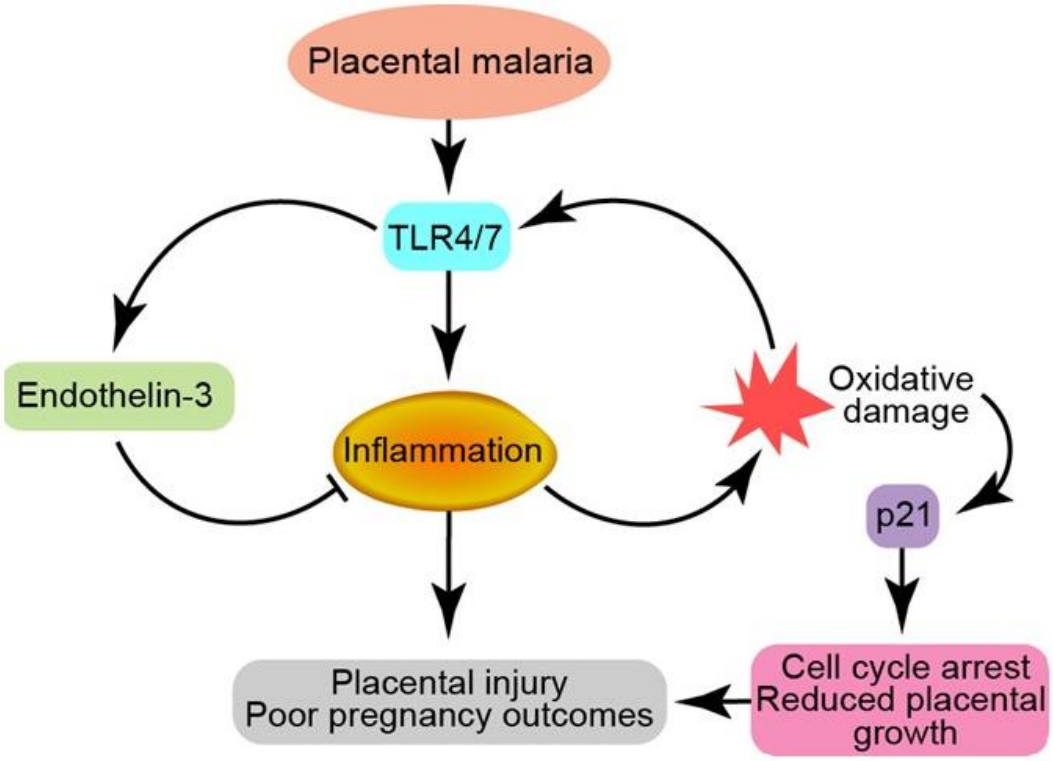

Figure 5. Schematic summary of the hypothesized TLR–Endothelin-3–oxidative stress axis in human placental malaria. Further investigation is needed to validate this axis and determine its potential for intervention against placental malaria.

471 **Tables**

472 **Table 1. Summary of placenta donors' demographics**

Group	Age (range)	Gravidity (range)	BW (g) (range)	PW (g) (range)	BW:PW (range)
All donors	24.7 years (18–40)	2.69 (1–7)	3077.64 (1600–5000)	479.04 (280.37–715)	6.51 (3.2–10.2)
MiP	24.4 years (18–30)	2.5 (1–7)	2870.5 (1600–5000) ****	464.3 (280.4–675) **	6.27 (3.2–10.2) *
NoMiP	25 years (18–40)	2.9 (1–7)	3272.3 (2000–4500)	492.9 (342.3–715)	6.73 (4.18–10)

473 Analysis of the placenta donors' data revealed that maternal age and gravidity were not
474 significantly different in the MiP (group with known history of malaria in pregnancy) vs the NoMiP
475 (group without known MiP history) groups (P = 0.45 and 0.12, respectively), whereas birthweight
476 (BW), placental weight (PW), and BW:PW ratios were significantly lower in the MiP group when
477 compared with the No MiP group (P = < 0.0001, 0.009, and 0.03, respectively). *, **, and ****
478 indicate $P < 0.05$, 0.005, and 0.0005, respectively.

479 **Table 2. Main characteristics of the donated placentas**

No. of placenta samples	179
Placental malaria-positive placenta samples (infected erythrocytes present)	92 (51.4%)
Placental malaria-negative placenta samples	58 (32.4%)
Placentas with past placental malaria infection (hemozoin present)	29 (16.2%)
Placenta samples from male fetuses	92 (51.4%)
Placenta samples from female fetuses	87 (48.6 %)
Placenta samples from pre-term deliveries	8 (4.5%)

480 The general characteristics of the placentas used in this study are summarized. Placental malaria
481 status was determined using hematoxylin and eosin (H&E) analysis.

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483

484 **Table 3. List of primers used in the study**

Target	Forward primer	Reverse primer
TLR4	5'-AGACCTGTCCCTGAACCCTAT-3'	5'-CGATGGACTTCTAAACCAGCCA-3'
TLR9	5'-CTGCCTTCCTACCCTGTGAG-3'	5'-GGATGCGGTTGGAGGACAA-3'
TLR7	5'-TCCTTGGGGCTAGATGGTTTC-3'	5'-TCCACGATCACATGGTTCTTTG-3'
β-actin	5'-CATGTACGTTGCTATCCAGGC-3'	5'-CTCCTTAATGTCACGCACGAT-3'
p21	5'-TGTCCGTCAGAACCCATGC-3'	5'-AAAGTCGAAGTTCCATCGCTC-3'
Endothelin-3	5'-GGGACTGTGAAGAGACTGTGG-3'	5'-AGACACACTCCTTGTCCTTGTA-3'
P. falciparum	5′-TTAAACTGGTTTGGGAAACCAAATATATT-3′	5′-ACACAATGAACTCAATCATGACTACCCGTC-3′

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499 **References**

500 [1] World Health Organization. World malaria report 2023. 2023.

501 [2] Rogerson SJ, Mwapasa V, Meshnick SR. Malaria in pregnancy: Linking immunity and
502 pathogenesis to prevention. American Journal of Tropical Medicine and Hygiene
503 2007;77:14–22.

504 [3] Bauserman M, Conroy AL, North K, Patterson J, Bose C, Meshnick S. An Overview of
505 Malaria in Pregnancy. Semin Perinatol 2019;43:282.
506 https://doi.org/10.1053/J.SEMPERI.2019.03.018.

507 [4] Fried M, Kurtis JD, Swihart B, Pond-Tor S, Barry A, Sidibe Y, et al. Systemic Inflammatory
508 Response to Malaria During Pregnancy Is Associated With Pregnancy Loss and Preterm
509 Delivery. Clin Infect Dis 2017;65:1729. https://doi.org/10.1093/CID/CIX623.

510 [5] Schmiegelow C, Matondo S, Minja DTR, Resende M, Pehrson C, Nielsen BB, et al.
511 Plasmodium falciparum Infection Early in Pregnancy has Profound Consequences for Fetal
512 Growth. J Infect Dis 2017;216:1601–10. https://doi.org/10.1093/infdis/jix530.

513 [6] Trampuz A, Jereb M, Muzlovic I, Prabhu RM. Clinical review: Severe malaria. Crit Care
514 2003;7:315. https://doi.org/10.1186/CC2183.

515 [7] Ayres Pereira M, Mandel Clausen T, Pehrson C, Mao Y, Resende M, Daugaard M, et al.
516 Placental Sequestration of Plasmodium falciparum Malaria Parasites Is Mediated by the
517 Interaction Between VAR2CSA and Chondroitin Sulfate A on Syndecan-1. PLoS Pathog
518 2016;12:e1005831. https://doi.org/10.1371/journal.ppat.1005831.

519 [8] Frank HG. Placental Development. Fetal and Neonatal Physiology, 2-Volume Set
520 2017:101–13. https://doi.org/10.1016/B978-0-323-35214-7.00010-X.

521 [9] Harrington WE, Duffy PE. Congenital malaria: Rare but potentially fatal. Ped Health
522 2008;2:235–48.
523 https://doi.org/10.2217/17455111.2.2.235/ASSET/IMAGES/LARGE/GRAPHIC22.JPEG.

524 [10] Fried M, Duffy PE. Malaria during Pregnancy. Cold Spring Harb Perspect Med
525 2017;7:a025551. https://doi.org/10.1101/cshperspect.a025551.

526 [11] Rogerson SJ, Brown HC, Pollina E, Abrams ET, Tadesse E, Lema VM, et al. Placental
527 Tumor Necrosis Factor Alpha but Not Gamma Interferon Is Associated with Placental
528 Malaria and Low Birth Weight in Malawian Women. Infect Immun 2003;71:267.
529 https://doi.org/10.1128/IAI.71.1.267-270.2003.

530 [12] Zakama AK, Ozarslan N, Gaw SL. Placental Malaria. Curr Trop Med Rep 2020;7:162–71.
531 https://doi.org/10.1007/S40475-020-00213-2.

532 [13] Kobia FM, Maiti K, Obimbo MM, Smith R, Gitaka J. Potential pharmacologic interventions
533 targeting TLR signaling in placental malaria. Trends Parasitol 2022;38:513–24.
534 https://doi.org/10.1016/J.PT.2022.04.002.

535 [14] Rodrigues-Duarte L, Pandya Y, Neres R, Penha-Gonçalves C. Fetal and maternal innate
536 immunity receptors have opposing effects on the severity of experimental malaria in
537 pregnancy: Beneficial roles for fetus-derived Toll-like receptor 4 and type I interferon
538 receptor 1. Infect Immun 2018;86. https://doi.org/10.1128/IAI.00708-
539 17/SUPPL_FILE/ZII999092383S1.PDF.

540 [15] Pandya Y, Marta A, Barateiro A, Bandeira CL, Dombrowski JG, Costa J, et al. TLR4-
541 Endothelin Axis Controls Syncytiotrophoblast Motility and Confers Fetal Protection in
542 Placental Malaria. Infect Immun 2021;89. https://doi.org/10.1128/IAI.00809-
543 20/ASSET/283CE60E-5A6D-46F3-9439-
544 7A889F86217A/ASSETS/IMAGES/LARGE/IAI.00809-20-F001.JPG.

545 [16] Vasquez M, Zuniga M, Rodriguez A. Oxidative Stress and Pathogenesis in Malaria. Front
546 Cell Infect Microbiol 2021;11. https://doi.org/10.3389/FCIMB.2021.768182.

547 [17] Tawadros PS, Powers KA, Ailenberg M, Birch SE, Marshall JC, Szaszi K, et al. Oxidative
548 Stress Increases Surface Toll-Like Receptor 4 Expression in Murine Macrophages Via
549 Ceramide Generation. Shock 2015;44:157–65.
550 https://doi.org/10.1097/SHK.0000000000000392.

551 [18] Harberts E, Gaspari AA. TLR Signaling and DNA Repair: Are They Associated? J Invest
552 Dermatol 2013;133:296. https://doi.org/10.1038/JID.2012.288.

553 [19] Canene-Adams K. Preparation of Formalin-fixed Paraffin-embedded Tissue for
554 Immunohistochemistry. Methods Enzymol 2013;533:225–33.
555 https://doi.org/10.1016/B978-0-12-420067-8.00015-5.

556 [20] Senagore PK, Holzman CB, Parks WT, Catov JM. Working towards a Reproducible Method
557 for Quantifying Placental Syncytial Knots. Http://DxDoiOrg/102350/15-08-1701-OA1
558 2016;19:389–400. https://doi.org/10.2350/15-08-1701-OA.1.

559 [21] Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time
560 Quantitative PCR and the 2−ΔΔCT Method. Methods 2001;25:402–8.
561 https://doi.org/10.1006/METH.2001.1262.

562 [22] Hayward CE, Lean S, Sibley CP, Jones RL, Wareing M, Greenwood SL, et al. Placental
563 adaptation: What can we learn from Birthweight:placental weight ratio? Front Physiol
564 2016;7:177721. https://doi.org/10.3389/FPHYS.2016.00028/BIBTEX.

565 [23] Zhang Y, Zhu X, Feng Y, Pang W, Qi Z, Cui L, et al. TLR4 and TLR9 signals stimulate
566 protective immunity against blood-stage Plasmodium yoelii infection in mice. Exp Parasitol
567 2016;170:73–81. https://doi.org/10.1016/J.EXPPARA.2016.09.003.

568 [24] Baccarella A, Fontana MF, Chen EC, Kim CC. Toll-Like Receptor 7 Mediates Early Innate
569 Immune Responses to Malaria. Infect Immun 2013;81:4431.
570 https://doi.org/10.1128/IAI.00923-13.

571 [25] Barboza R, Lima FA, Reis AS, Murillo OJ, Peixoto EPM, Bandeira CL, et al. TLR4-Mediated
572 Placental Pathology and Pregnancy Outcome in Experimental Malaria. Sci Rep 2017;7.
573 https://doi.org/10.1038/S41598-017-08299-X.

574 [26] Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2' -deoxyguanosine (8-OHdG): A
575 critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ
576 Carcinog Ecotoxicol Rev 2009;27:120–39. https://doi.org/10.1080/10590500902885684.

577 [27] Villeneuve NF, Sun Z, Chen W, Zhang DD. Nrf2 and p21 regulate the fine balance between
578 life and death by controlling ROS levels. Cell Cycle 2009;8:3255.
579 https://doi.org/10.4161/CC.8.20.9565.

580 [28] Salgado C, Ayodo G, Macklin MD, Gould MP, Nallandhighal S, Odhiambo EO, et al. The
581 prevalence and density of asymptomatic Plasmodium falciparum infections among children
582 and adults in three communities of western Kenya. Malar J 2021;20.
583 https://doi.org/10.1186/S12936-021-03905-W.

584 [29] Idris ZM, Chan CW, Kongere J, Gitaka J, Logedi J, Omar A, et al. High and Heterogeneous
585 Prevalence of Asymptomatic and Sub-microscopic Malaria Infections on Islands in Lake
586 Victoria, Kenya. Scientific Reports 2016 6:1 2016;6:1–13.
587 https://doi.org/10.1038/srep36958.

588 [30] Menendez C, Ordi J, Ismail MR, Ventura PJ, Aponte JJ, Kahigwa E, et al. The Impact of
589 Placental Malaria on Gestational Age and Birth Weight. J Infect Dis 2000;181:1740–5.
590 https://doi.org/10.1086/315449.

591 [31] Crocker IP, Tanner OM, Myers JE, Bulmer JN, Walraven G, Baker PN. Syncytiotrophoblast
592 degradation and the pathophysiology of the malaria-infected placenta. Placenta
593 2004;25:273–82. https://doi.org/10.1016/j.placenta.2003.09.010.

594 [32] Lampi K, Papadogiannakis N, Sirotkina M, Pettersson K, Ajne G. Massive perivillous fibrin
595 deposition of the placenta and pregnancy outcome: A retrospective observational study.
596 Placenta 2022;117:213–8. https://doi.org/10.1016/J.PLACENTA.2021.12.013.

597 [33] Brink LT, Roberts DJ, Wright CA, Nel DG, Schubert PT, Boyd TK, et al. Placental pathology
598 in spontaneous and iatrogenic preterm birth: Different entities with unique pathologic
599 features. Placenta 2022;126:54. https://doi.org/10.1016/J.PLACENTA.2022.06.004.

600 [34] Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003;21:335–76.
601 https://doi.org/10.1146/annurev.immunol.21.120601.141126.

602 [35] Pandya Y, Penha-Gonçalves C. Maternal-fetal conflict during infection: Lessons from a
603 mouse model of placental malaria. Front Microbiol 2019;10:1126.
604 https://doi.org/10.3389/FMICB.2019.01126/BIBTEX.

605 [36] Fukata M, Vamadevan AS, Abreu MT. Toll-like receptors (TLRs) and Nod-like receptors
606 (NLRs) in inflammatory disorders. Semin Immunol 2009;21:242–53.
607 https://doi.org/10.1016/J.SMIM.2009.06.005.

608 [37] Chua CLL, Khoo SKM, Ong JLE, Ramireddi GK, Yeo TW, Teo A. Malaria in Pregnancy:
609 From Placental Infection to Its Abnormal Development and Damage. Front Microbiol
610 2021;12:3452. https://doi.org/10.3389/FMICB.2021.777343/BIBTEX.

611 [38] Rodríguez-Pascual F, Busnadiego O, Lagares D, Lamas S. Role of endothelin in the
612 cardiovascular system. Pharmacol Res 2011;63:463–72.
613 https://doi.org/10.1016/J.PHRS.2011.01.014.

614 [39] Sato A, Ebina K. Common mechanism in endothelin-3 and PAF receptor function for anti-
615 inflammatory responses. Eur J Pharmacol 2013;718:30–3.
616 https://doi.org/10.1016/J.EJPHAR.2013.09.025.

617 [40] Sato A, Ebina K. Endothelin-3 at low concentrations attenuates inflammatory responses
618 via the endothelin B2 receptor. Inflamm Res 2013;62:417–24.
619 https://doi.org/10.1007/S00011-013-0594-3.

620 [41] Burton GJ, Jauniaux E. Oxidative stress. Best Pract Res Clin Obstet Gynaecol
621 2011;25:287. https://doi.org/10.1016/J.BPOBGYN.2010.10.016.

622 [42] Sarr D, Cooper CA, Bracken TC, Martinez-Uribe O, Nagy T, Moore JM. Oxidative Stress:
623 A Potential Therapeutic Target in Placental Malaria. Immunohorizons 2017;1:29.
624 https://doi.org/10.4049/IMMUNOHORIZONS.1700002.

625 [43] Hansen KD, Wu Z, Irizarry RA, Leek JT. Sequencing technology does not eliminate
626 biological variability. Nat Biotechnol 2011;29:572. https://doi.org/10.1038/NBT.1910.

627