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 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**

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 study
91 as described recently [19]. This study did not require participant written informed consent since it
92 was granted during biobanking [19]. Participants with a known record of sexually transmitted
93 disease infection during pregnancy, those with pregnancy-associated noncommunicable
94 diseases (preeclampsia and gestational diabetes) during the current pregnancy (at placenta
95 donation), and those with twin pregnancies were excluded from analyses. Based on the

96 underlying biobank data, malaria in pregnancy was defined as having at least one episode of
97 hospital-diagnosed malaria during pregnancy. All data underlying the databank were deidentified.
98 Equal numbers of male and female placentas were analyzed. The characteristics of the biobank's
99 placenta donors and samples are summarized in Table 1.

100 **2.2 Histological analysis**

101 Formalin-fixed placenta tissues were embedded in paraffin blocks as previously described [20]
102 using an automated tissue embedding system (MediMeas). H&E analysis was used to confirm
103 placental malaria presence, which is indicated by the presence of infected erythrocytes in the
104 placenta. Briefly, formalin-fixed paraffin-embedded samples were sectioned onto charged
105 microscope slides (Dako, Cat No. K8020) at a 5-μm thickness, dried at 37 °C overnight on a slide
106 warmer, dewaxed in xylene (Finar Chemicals, Cat No. 21940LC250), rehydrated by dipping
107 across an alcohol gradient of absolute, 95%, 70%, and 50% ethanol (Scharlau, Cat No.
108 ET00052500), and then in distilled water. They were then submerged in hematoxylin (Loba
109 Chemie, Cat No. 04023) for seven minutes, rinsed with running water, and then destained through
110 10 dips in acid alcohol (1% hydrochloric acid in 70% ethanol). Next, they were submerged in eosin
111 (Griffchem, Cat No. 45380) for 45 seconds followed by dehydration in 95% ethanol and absolute
112 ethanol (five minutes each) and then cleared in xylene baths (10 minutes each) before being
113 cover-slipped using dibutylphthalate polystyrene xylene mountant (Finar Chemicals, Cat No.
114 10525LM250). The slides were then examined under a microscope (Richter Optica UX1, M2
115 Scientifics), followed by imaging in ≥10 fields of view per slide at a 40X magnification using a
116 Moticam microscope camera (Motic Scientific). Placental malaria was then diagnosed as
117 described before [21], based on the presence of infected erythrocytes. Placental malaria-
118 associated histopathological features were assessed by counting the number of syncytial knots
119 as described previously [22], and measuring the fibrin-occupied placental areas using imageJ.
120 These analyses were done in at least 10 fields of view per sample. The levels of placental malaria

121 burden in the placental malaria-positive samples were determined by counting the number of
122 identified infected erythrocytes in at least 10 fields of view per slide imaged at a magnification of
123 40X.

124 **2.4 RNA extraction and reverse transcription quantitative PCR (RT-qPCR)**

125 Total RNA was extracted from placenta tissue using a HigherPurity™ Tissue Total RNA
126 Purification kit as per the manufacturer's guidelines (Canvax, cat No. AN0152) and quantified
127 using a NanoDrop Microvolume Spectrophotometer (ThermoFisher Scientific) following the
128 manufacturer's instructions. For each sample, 500 ng of RNA were retrotranscribed into cDNA
129 using a LunaScript™ RT SuperMix cDNA Synthesis Kit (NEB, Cat. No. E3010L) using the
130 manufacturer's protocol. RT-qPCR analysis was done on a QuantStudio™ 5 Real-Time PCR
131 System in a final volume of 20 µl containing 10 µl of GoTaq qPCR Master Mix (Promega, Cat No.
132 PRA6001), 2 µl of the forward plus reverse primers (final primer concentration: 500 nM), 3 µl of
133 nuclease free water (Promega, Cat No. P119E), and 5 µl of cDNA using the following program:
134 50 °C for two minutes, 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds and
135 60°C for 30 seconds. Relative gene expression was determined using the $2^{-\Delta\Delta ct}$ method [23], using
136 β-actin as the reference gene. Primers were purchased from Macrogen and primer sequences
137 are provided in Table 3.

138 **2.5 *P. falciparum* detection PCR**

139 The presence of *P. falciparum* in placenta samples was evaluated using One Taq® Quick-Load®
140 2X Master Mix with Standard Buffer (NEB, Cat No. M0486L) and the following thermocycler
141 program: Initial denaturation at 95 °C for five minutes, followed by 35 cycles of denaturation at 95
142 °C for 30 seconds, annealing at 55 °C for 60 seconds, and extension at 72 °C for 75 seconds,
143 and then a final extension at 72 °C for five minutes. Primer sequences are shown in Table 3. The
144 PCR product was subjected to agarose (Sigma–Aldrich, Cat No. A9539) gel electrophoresis using
145 1X tris–borate–EDTA buffer alongside a 100 base pair ladder using SafeView™ Classic (Applied

146 Biological Materials, Cat No. G108) nucleic acid stain and Gel Loading Dye, Purple (6X) (NEB,
147 Cat No. B7024S). The bands were developed and imaged using a UVITEC Gel Documentation
148 System (Cleaver Scientific).

149 **2.6 Immunohistochemistry**

150 The sections were deparaffinized by warming at 55 °C for 15 minutes followed by dipping in three
151 xylene baths, about 10 dips each. They were then rehydrated and subjected to heat-induced
152 epitope retrieval by boiling for 30 minutes in Citrate Buffer, pH 6.0 (Sigma–Aldrich, cat. No.
153 C9999). They were then cooled to room temperature, rinsed with distilled water for five minutes
154 and then blocked with 0.3% Triton-X in 1X phosphate-buffered saline (PBST). Next, they were
155 blocked with 10% normal donkey serum (Abcam, cat. No. ab7475) in PBST for two hours followed
156 by overnight incubation (4 °C) with anti-DNA/RNA damage antibody [15A3] (Abcam, cat. No.
157 ab62623) at 1:2500 in blocking solution. Sections were then washed thrice (10 minutes each)
158 using PBST and then incubated at room temperature for two hours with horseradish peroxidase-
159 conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, cat. No. 115-035-
160 003) at 1:5000 in blocking solution. The sections were then washed thrice (10 minutes each) using
161 PBST followed by signal development using an ImmPACT® DAB Substrate Kit (Vector, cat. No.
162 SK-4105) as per the manufacturer's protocol. They were then dehydrated using 95%, 95%, 100%,
163 and 100% ethanol (five minutes each), cleared by dipping in three xylene baths and then cover-
164 slipped using a xylene-based mountant and allowed to dry. They were then examined under a
165 light microscope and imaged at a magnification of 40X.

166 **2.7 Data analyses**

167 Statistical analyses were done using GraphPad Prism version 9. Data are presented as
168 percentages, raw values, or mean ± standard deviation. Differences between two groups were
169 compared using a t-test. Correlation analyses were done using nonparametric Spearman
170 correlation analysis. For each placenta, the placental malaria burden in placentas was indicated

171 by the total number of infected erythrocytes in the placenta section. The birthweight-to-placenta
172 weight ratio was obtained by dividing the birthweight (grams) with the corresponding placenta's
173 weight (grams). The correlation between placental malaria status and birthweight, placental
174 weight, and birthweight-to-placental weight ratio was assessed using GraphPad prism to examine
175 the impact of placental malaria on fetal outcomes. $P < 0.05$ indicates statistically significant
176 differences.

177 **3. Results**

178 **3.1 Main characteristics of the placenta donors and donated placentas**

179 All placenta donors were ≥18-years-old. The mean age, gravidity, birthweight, placental weight,
180 and birthweight-to-placental weight ratio of the cohort of placenta donors were 24.7 years, 2.69,
181 3077.64 g, 470.04 g, and 6.51, respectively (Table 1). Grouping the placenta donors into those
182 with a known history of malaria in pregnancy (MiP) and those without (NoMiP), revealed that in
183 the MiP vs NoMiP groups, maternal age and gravidity were not significantly different (mean age:
184 24.4 [range: 18–30] vs 25 [range: 18–40] years, $P = 0.45$; mean gravidity: 2.5 [range: 1–7] vs 2.9
185 [range: 1–7), $P = 0.12$). However, in the MiP vs NoMiP groups, birthweight (mean: 2870.5 [range:
186 1600–5000] vs 3272.3 [range: 2000–4500] g, placental weight (mean: 464.3 [range: 280.4–675)
187 vs 492.9 [range: 342.3–715] g, and birthweight-to-placental weight ratio (mean: 6.27 [range: 3.2–
188 10.2] vs 6.73 [range: 4.18–10], were significantly lower in the MiP group ($P < 0.0001$, = 0.009,
189 and = 0.03, respectively). The presence of placental malaria was determined using H&E analysis
190 as we described recently [19]. Of the placentas, 92 (51.4%) belonged to male babies, and eight
191 (4.5%) were preterm (Table 2). All downstream analyses were done on placenta samples that
192 were confirmed to be placental malaria-positive or placental malaria-negative using H&E staining.

193 **3.2 Placental malaria correlates negatively with birthweight and birthweight-to-placenta**
194 **ratio**

195 Representative images of placental malaria-negative and placental malaria-positive samples are
196 shown in Figure 1A–B, and the presence of *P. falciparum* in placental malaria-positive tissues
197 was confirmed using PCR (Figure 1C). Analysis of the data underlying the placental biobank
198 revealed that when compared with the placental malaria-negative group, birthweight was
199 significantly lower in the placental malaria-positive group, which had more low birthweight cases
200 (Figure 1A, $P = 0.03$, low birthweight: <2500 g), but placental weight did not differ between the
201 two groups (Figure 2B, $P = 0.8$). However, the birthweight-to-placental weight ratio was lower in
202 the placental malaria-positive group, although the difference did not reach statistical significance
203 (Figure 1F, $P = 0.1$). Next, we used the H&E images to determine the proportion (%) of infected
204 erythrocytes (IEs) in each placenta sample, and then used the obtained values to assess the
205 correlation between the placental malaria burden and birthweight, placental weight, and the
206 birthweight-to-placental weight ratio, i.e., the fetal weight obtained per gram of the placenta, which
207 is an indicator of placental efficiency, with higher birthweight-to-placental weight ratios indicating
208 greater placental efficiency [24]. This analysis revealed negative correlation between IEs
209 (proportion of infected erythrocytes [%]) and birthweight (correlation coefficient [rs]: -0.23, $P <$
210 0.005, 95% confidence interval [CI]: -0.37 – -0.08), and IEs (%) and birthweight-to-placental
211 weight ratio (rs: -0.20, $P = 0.007$, 95% CI: -0.35 – -0.05). Expectedly, placenta weight had a
212 positive correlation with birthweight (rs: 0.28, $P < 0.001$, 95% CI: 0.13 – 0.41) and a negative
213 correlation with birthweight-to-placental weight ratio (rs: -0.42, $P < 0.001$, 95% CI: -0.54 – -0.28).
214 However, placental malaria burden did not exhibit correlation with placenta weight (rs: 0.01, $P =$
215 0.93, 95% CI: -0.16 – 0.15). Taken together, these findings indicate that placental malaria impairs
216 placenta function, leading to low birthweight via reduced placenta efficiency, as indicated by the
217 negative correlation between placental malaria burden and the birthweight-to-placental weight
218 ratios of placental malaria-exposed neonates.

219 **3.3 Placental malaria markedly alters placental histological features**

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

229 **3.4 Placental malaria is associated with an upregulation of TLR4, TLR7, and Endothelin 3**

230 We then sought to determine if placental malaria altered the expression of TLRs. To this end, we
231 focused on TLR4, TLR7, and TLR9, which have been associated with response to malaria
232 infection in mice [25,26], and with mouse placental malaria in the case of TLR4 [27], although this
233 has not been reported in human placental malaria. To evaluate the effect of placental malaria on
234 these innate immune system receptors, we assessed their expression levels using RT-qPCR. The
235 analysis revealed that when compared with placental malaria-negative controls, placental
236 malaria-positive samples expressed significantly higher levels of TLR4 and TLR7, but not TLR9
237 (Figure 3A–C, $P = 0.002$, 0.03, and 0.59, respectively). This is consistent with mouse data
238 showing that placental malaria upregulates TLR4-mediated expression of endothelin-1 [15]. We
239 therefore wondered if human placental malaria alters the expression of Endothelin genes. RT-
240 qPCR analysis of Endothelin-1 and -3 gene expression revealed that only Endothelin-3 was
241 detectable in our placenta samples and that when compared with placental malaria-negative
242 placentas, placental malaria-positive samples had significantly higher Endothelin-3 levels (Figure
243 3D, $P = 0.004$), indicating the presence of a TLR–Endothelin signaling axis in response to human
244 placental malaria.

245 **3.5 Placental malaria is associated with high oxidative DNA damage**

246 Since TLR4 was upregulated in placental malaria-positive placenta samples, we wondered
247 whether placental malaria-driven TLR4 activation is associated with a dysregulation of other
248 signaling processes that may underlie or contribute to placental malaria-mediated placental
249 pathobiology. Because malaria is known to be strongly inflammatory and oxidative, which may
250 drive host tissue damage [16], and because oxidative DNA damage is associated with TLR4
251 upregulation [17] and that TLR signaling is thought to promote DNA repair [18], we wondered if
252 the TLR4 upregulation in the placental malaria-positive samples might be associated with
253 placental oxidative DNA damage. To assess this possibility, we used immunohistochemistry to
254 assess the levels of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative DNA stress [28]. This
255 analysis revealed that when compared with placental malaria-negative samples, placental
256 malaria-positive samples express markedly higher levels of 8-hydroxy-2'-deoxyguanosine (Figure
257 4A). Staining the same samples with the secondary antibody only (without the primary antibody)
258 confirmed signal specificity (Figure 4B). To further assess the effect of placental malaria on
259 oxidative stress, we used RT-qPCR to examine the level of p21, a mediator of cell cycle arrest
260 and indicator of cellular response to DNA damage [29]. This revealed that when compared with
261 placental malaria-negative samples, placental malaria-positive samples had significantly higher
262 levels of p21 (Figure 4C, P = 0.02). Taken together, these data indicate that placental malaria
263 triggers markedly high levels of placental oxidative DNA stress, placenta tissue damage, and
264 cellular response to DNA stress, which may contribute to placental malaria pathobiology, and that
265 in response, TLR signaling may at least in part, be upregulated to counter these adverse effects
266 through the promotion of DNA repair.

267 **4. Discussion**

268 Malaria in pregnancy often results in placental malaria, where erythrocytes that are infected with
269 *P. falciparum*, the parasite that most frequently causes placental malaria, sequester in placental

270 intervillous spaces [7]. Placental malaria may cause various adverse fetal outcomes, including
271 stillbirth, preterm birth, and fetal growth restriction [3–5], and because *P. falciparum* rarely
272 undergoes vertical transmission [9], these effects are likely caused by placental malaria-driven
273 pathobiological effects in the placenta, which may impair placental function. However, although
274 studies have implicated effects like inflammation and histological changes in placental malaria
275 pathogenesis, the mechanisms underlying the adverse effects of human placental malaria on the
276 placenta are unclear. Moreover, because many malaria in pregnancy cases in malaria-endemic
277 regions are asymptomatic [30,31] and the placenta is inaccessible during pregnancy, there are
278 no ways of detecting and intervening against placental malaria during pregnancy. Thus, there is
279 an urgent need to better understand the placental pathobiology of placental malaria to guide the
280 development of effective diagnostic and therapeutic tools.

281 Mouse models indicate that placental malaria triggers innate immune responses (mainly via
282 TLR4) that are associated with poor fetal outcomes and that TLR4-mediated fetal responses to
283 placental malaria cause improved outcomes [14]. However, the effect of human placental malaria
284 on TLR-mediated immunity in the placenta has not been examined. In this study, we leveraged
285 our well-characterized biobank of placenta samples from a malaria endemic region of Kenya
286 (Table 1) and found that in our study cohort, placental malaria burden had a significant negative
287 correlation with birthweight and birthweight-to-placental weight ratio, that it was associated with
288 significantly higher placental histological lesions, higher levels of TLR4 and Endothelin-3
289 expression, and enhanced oxidative DNA damage when compared with samples without
290 placental malaria.

291 Consistent with previous findings implicating placental malaria in fetal growth restriction [32], we
292 observed that in our study cohort, relative to the placental malaria-negative cases, placental
293 malaria was associated with low birthweight. Moreover, we observed that placental malaria was
294 associated with lower birthweight-to-placental weight ratios, an indicator of placental efficiency in

295 which higher ratios indicate higher nutrient transfer for every gram of the placenta and vice versa
296 [24], but not with lower placental weight (Figure 1D–F), an observation that to our knowledge, has
297 not been previously reported in human placental malaria. Interestingly, our analyses also indicate
298 that the placental malaria burden (percentage of infected erythrocytes in a sample's intervillous
299 spaces) correlates negatively with birthweight but not with placental weight. Taken together, these
300 observations indicate that placental malaria contributes to fetal growth restriction primarily by
301 impairing placental function, and not via placental growth inhibition, although the precise
302 mechanisms remain unclear. This possibility is crucial considering that women in malaria-endemic
303 regions may experience multiple malaria reinfections throughout pregnancy. Therefore, further
304 studies, such as using *in vitro* and organoid systems, are needed to comprehensively investigate
305 this possibility.

306 Our analyses revealed that placental malaria-positive samples had markedly higher levels of fibrin
307 deposition and syncytial knotting than placental malaria-negative samples, which is in line with
308 earlier findings [33]. These changes, which indicate placental injury, and have been associated
309 with placental malperfusion and poor fetal outcomes, including fetal growth restriction [34,35],
310 may contribute to the low birthweight observed in our placental malaria cohort. However, studies
311 are needed to establish the mechanisms by which placental malaria alters placental histological
312 features, how these changes correlate with fetal outcomes and postnatal wellbeing, and whether
313 they can predict fetal wellbeing in postnatal life.

314 TLRs are key innate immunity factors that sense host invasion by pathogens and activate host
315 immune defenses [36]. Mouse models of malaria indicate that TLR4, TLR7, and TLR9 are
316 involved in detecting and responding to malaria infection [25,26]. Moreover, mouse models
317 indicate that at the fetal–maternal interface, placental malaria activates TLR4-mediated immune
318 responses that drive poor fetal outcomes, and that fetal TLR4-mediated counterresponses
319 improve pregnancy outcomes [14,27,37]. However, this observation has not been previously

320 made in human placental malaria. Here, we observed that the expression levels of TLR4 and
321 TLR7, but not TLR9, were significantly upregulated in placental malaria-positive samples,
322 indicating that placental infection triggers an innate immune reaction and that it is mainly driven
323 by TLR4 and TLR7, although the status of other TLRs during placental malaria warrants
324 investigation. Considering that TLRs are important drivers of inflammation [38], which is
325 implicated in placental malaria pathogenesis [39], taken together with the observed changes in
326 placental histological features, our findings indicate for the first time, that TLR-mediated
327 responses to placental malaria may contribute to local placental inflammation, which may underlie
328 the observed placental malaria-associated low birthweight and placental weight, although the
329 precise mechanisms remain unclear. Mouse data show that placental malaria-driven TLR4
330 expression drives placental endothelin-1 expression [15], and for the first time, our findings show
331 that placental malaria is also associated with the upregulation of Endothelin-3. The Endothelin
332 ligands 1, 2, and 3 are a family of vasoactive factors that influence a range of cellular processes,
333 such as vascular remodeling and angiogenesis [40]. Moreover, Endothelin-3 is reported to have
334 anti-inflammatory effects [41,42], suggesting that its upregulation in the context of placental
335 malaria-mediated TLR4 upregulation is a mechanism of countering TLR4-dependent placenta
336 inflammation. Collectively, these observations indicate that human placental malaria may activate
337 a TLR4–Endothelin-3 signaling axis, but further studies are needed to test this hypothesis and to
338 determine its implications in placental malaria pathobiology and fetal outcomes.

339 Based on reports that malaria is strongly oxidative [16], oxidative stress causes tissue damage
340 [43], oxidative DNA damage upregulates TLR4 [17], and that the TLR pathway might promote
341 DNA repair [18], we reasoned that our observation of TLR4 and TLR7 upregulation in placental
342 malaria-positive samples might be accompanied by placental oxidative DNA damage. This
343 hypothesis was confirmed by our immunohistochemistry data, which showed that 8-hydroxy-2'-
344 deoxyguanosine, a marker of oxidative DNA stress [28], was markedly upregulated in placental

345 malaria samples. Moreover, gene expression analysis revealed that these events were
346 accompanied by a significant upregulation of p21, a cell cycle inhibitor and marker of cellular
347 response to DNA damage [29]. These observations align with previous findings that in a mouse
348 model, placental malaria is associated with placental oxidative damage [44]. Furthermore, p21
349 upregulation in the placenta may arrest the cell cycle to allow for oxidative damage resolution,
350 which may contribute to the low placental weight observed in our cohort, but this possibility
351 requires further investigation. Together, our data suggest the presence of a previously unknown
352 TLR–Endothelin–oxidative damage axis in human placental malaria.

353 Finally, our data also show that in the placental malaria-negative and placental malaria-positive
354 groups, the analyzed parameters varied across samples, which may reflect inter-individual
355 biological variability [45]. In the future, our observations will be validated experimentally, such as
356 by challenging cultured primary human trophoblasts with *P. falciparum* for detailed mechanistic
357 interrogation.

358 **5. Conclusion**

359 Despite its heavy burden and adverse effects on maternal and fetal outcomes, the mechanisms
360 underlying the placental pathobiology of placental malaria are unclear. Considering that malaria
361 is rarely transmitted to the fetus [9], the adverse fetal outcomes of malaria in pregnancy are mainly
362 driven by events that disrupt placenta physiology and function. Importantly, because of placental
363 inaccessibility, placental malaria can only be confirmed via postnatal placental histopathology.
364 These challenges highlight the urgent need to better understand the mechanisms underlying the
365 placental pathobiology of placental malaria, which may inform the development of sensitive tools
366 for diagnosing placental malaria during pregnancy as well as effective therapeutic interventions.
367 Our findings that placental malaria may drive TLR-mediated responses in the placenta raise the
368 possibility that modulating innate responses to placental malaria may improve fetal outcomes, as
369 we previously discussed [13]. Moreover, our identification of an axis involving TLRs, Endothelins,

370 and oxidative DNA damage during placental malaria (Figure 5) highlights processes with the
371 potential for intervention against human placental malaria. However, further studies, such as using
372 primary human trophoblasts, human placental organoids, or human placental *ex vivo* systems are
373 needed to validate our observations. Such approaches can investigate the mechanisms of
374 placental malaria pathobiology more rigorously than can be done using term placentas.

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386 **Conflict of interest**

387 The authors declare no conflicts of interest.

388 **Ethics statement**

389 This study was approved by Mount Kenya University's ethics review committee (approval number
390 2516).

391 **Data accessibility**

392 The data underlying this study are contained in the article.

Figures

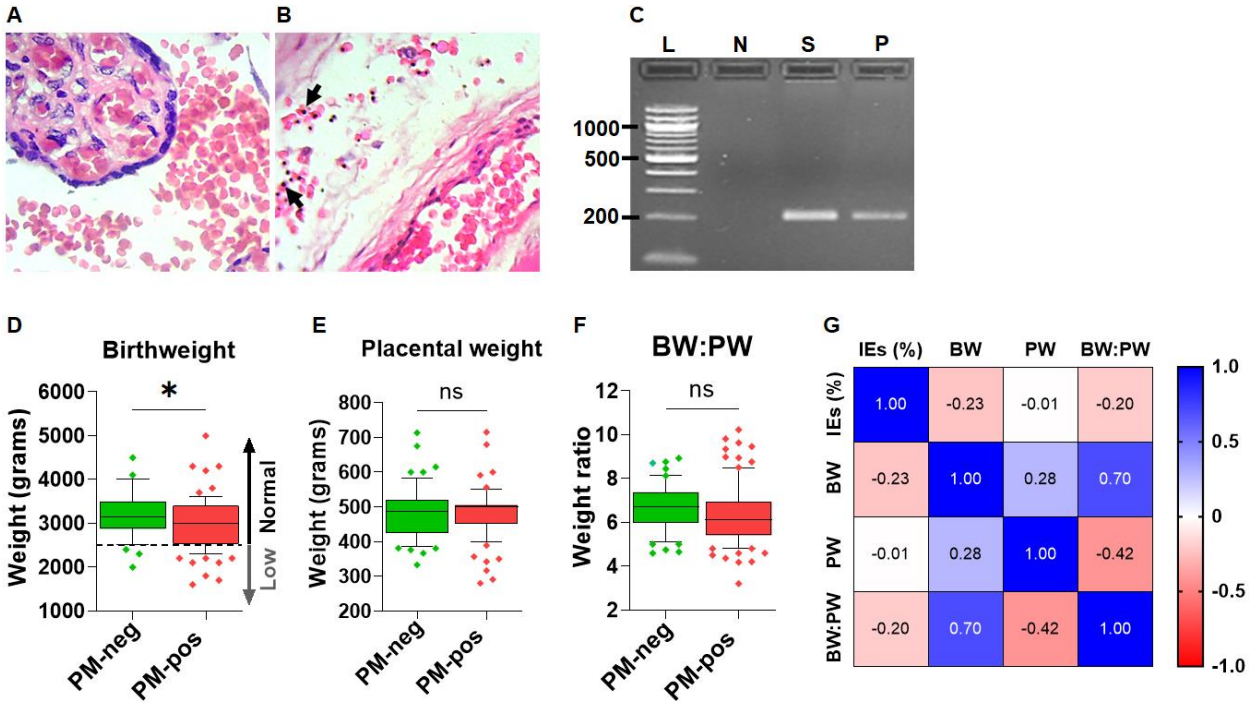
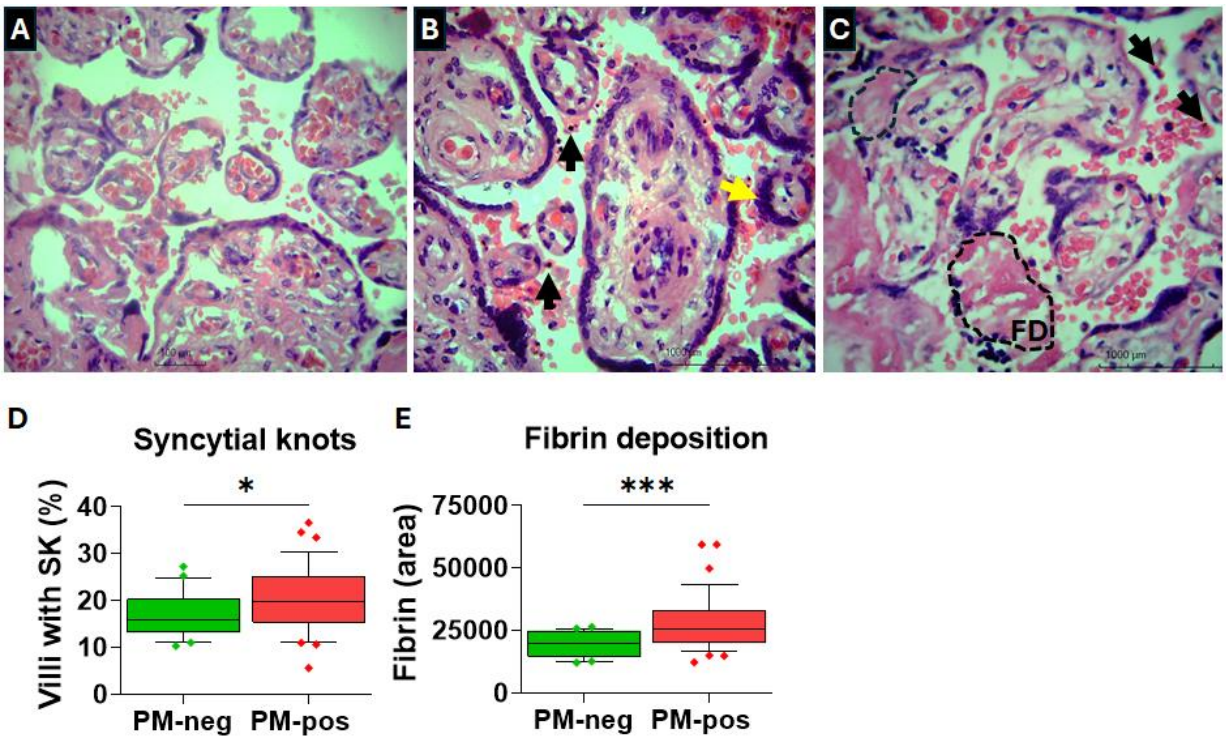


Figure 1. (A–B) Representative hematoxylin and eosin images of placental malaria (PM)-negative (A) and PM-positive samples (B). When compared with a PM-negative sample, the positive sample has malaria-infected erythrocytes (black arrowheads) in the placental intervillous space. (C) PCR confirmed the presence *of P. falciparum* in the PM-positive sample in B. L: 100 base pair ladder, N: PM-negative sample, S: PM-positive sample in B (histology), P: positive control (*P. falciparum* strain 3D7 genomic DNA). Expected PCR band size: 205 base pairs. (D–F) PM was associated with lower birthweight (BW) (D, P = 0.03) and lower birthweight-to-placental weight (BW:PW) ratio, although the difference did not reach statistical significance (F, P = 0.1), but not with lower placental weight (PW) (E, P = 0.8). In D–F, whiskers are drawn from the 10th to 90th percentile. (G) A correlation matrix shows that the proportion of infected erythrocytes, IEs (%), in the placenta correlated negatively with BW (P < 0.005) and the BW:PW ratio (P = 0.007), but it did not correlate with PW (P = 0.94). PW correlated positively with BW and negatively with the BW:PW ratio (both P < 0.001).



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

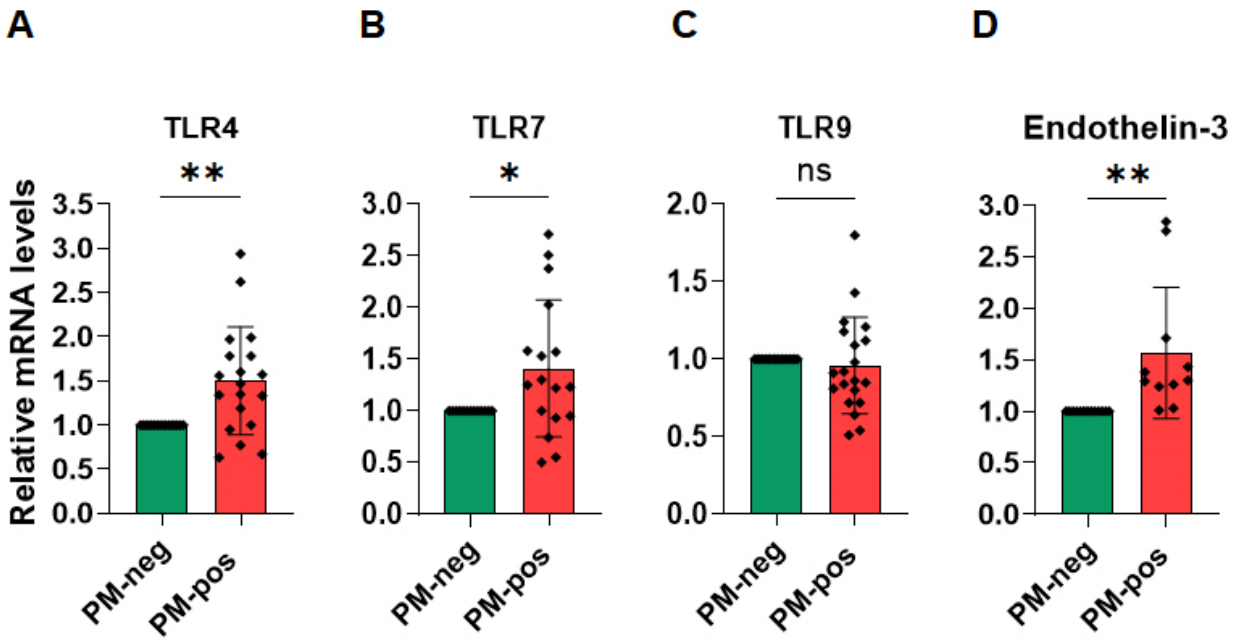

Figure 3. Placental malaria is associated with the upregulation of TLR4, TLR7, and Endothelin-3. (A–C) When compared with placental malaria (PM)-negative samples (PM-neg), PM-positive (PM-pos) samples expressed significantly higher levels of TLR4 and TLR7, but not TLR9 (P = 0.002, 0.03, and 0.59, respectively).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

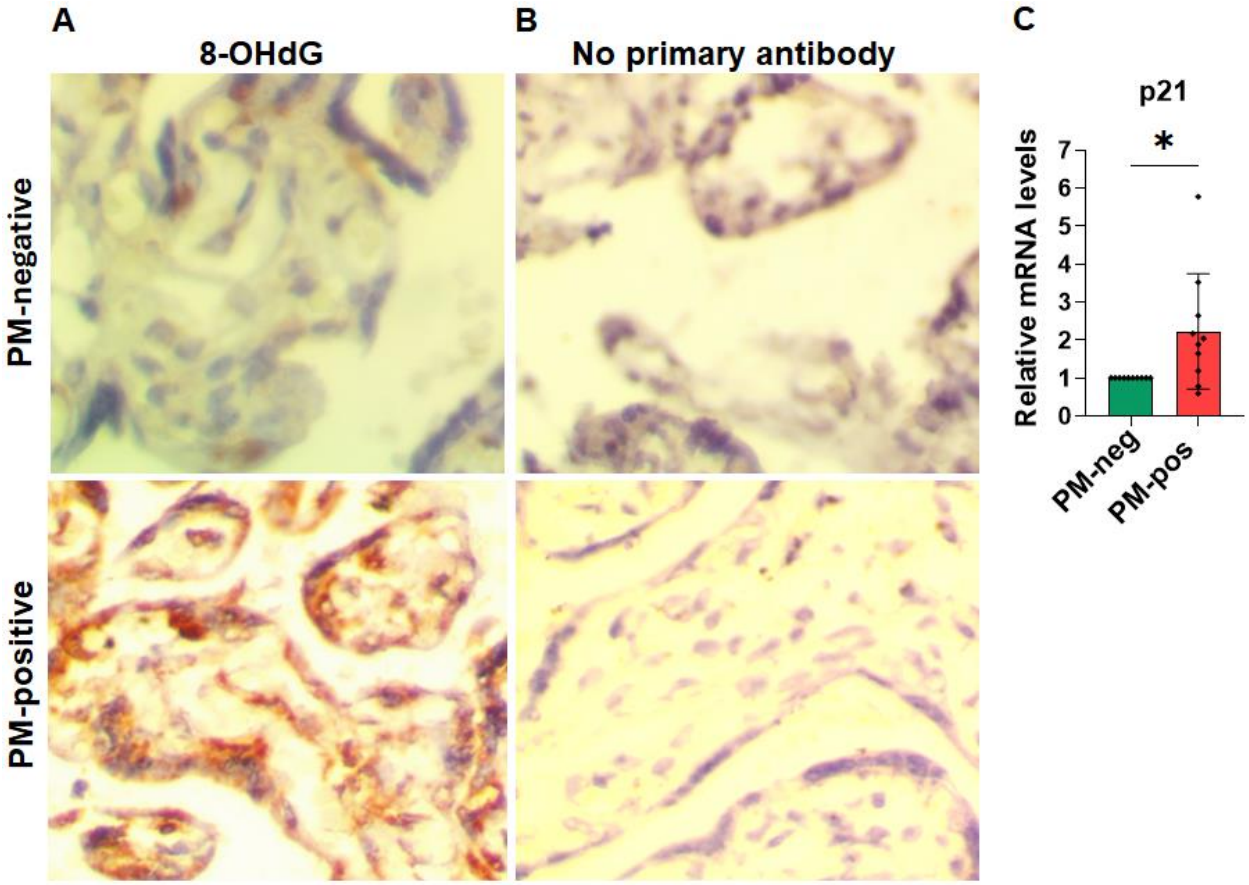

Figure 4. Analysis of oxidative DNA damage in placental malaria (PM)-negative vs PM-positive samples. (A–B) Immunohistochemistry revealed that when compared with PM-negative samples, PM-positive tissues had markedly higher levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), an indicator of oxidative damage.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

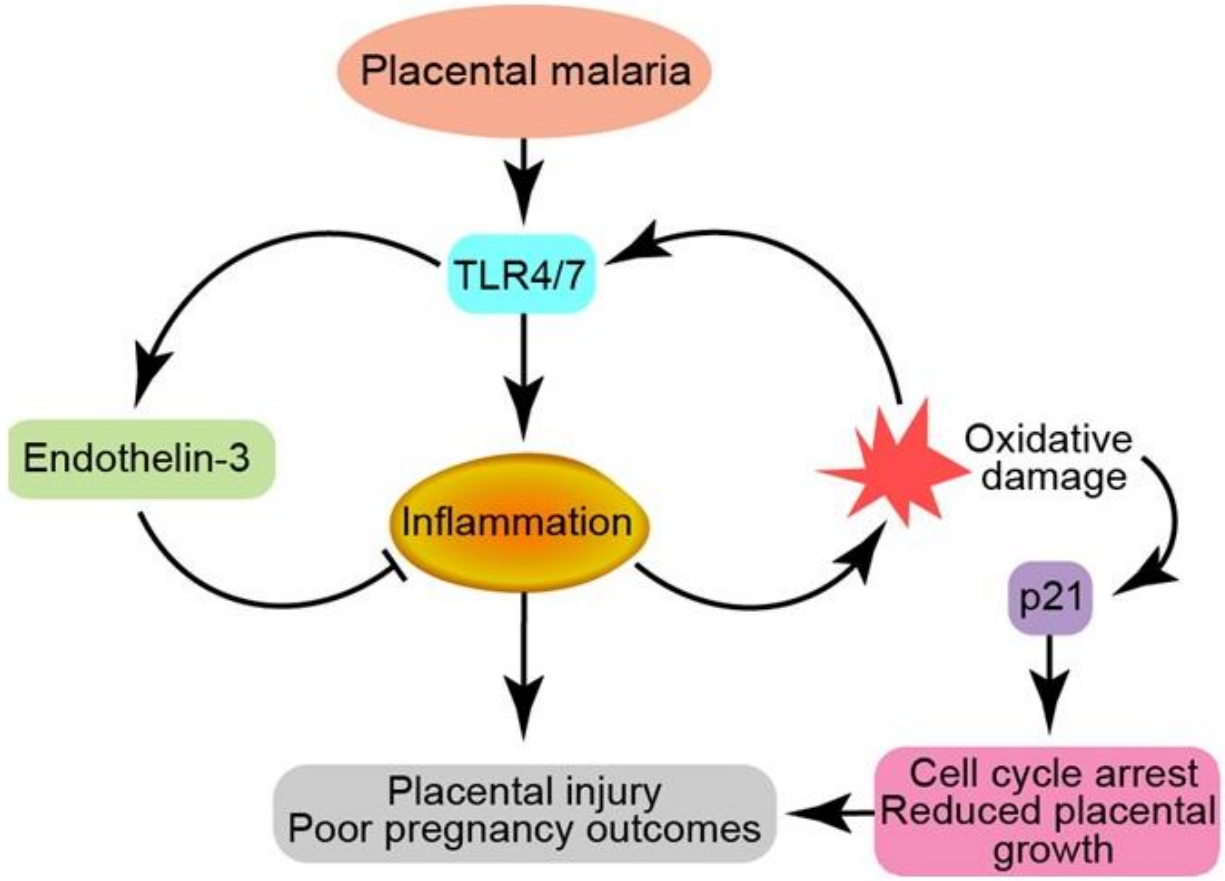

Figure 5. Schematic summary of the hypothesized TLR–Endothelin-3–oxidative stress axis in human placental malaria. Further investigation is needed to validate this axis and determine its potential for intervention against placental malaria.

442 **Tables**

443 **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)

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

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

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%)

451 The general characteristics of the placentas used in this study are summarized. Placental malaria
452 status was determined using hematoxylin and eosin (H&E) staining of placental tissue. Placental
453 malaria: observation of at least one infected erythrocyte [46]. Past placental malaria: absence of
454 infected erythrocytes coupled with hemozoin in fibrin and pigmented macrophages [21]. Chronic
455 placental malaria: detection of infected erythrocytes, as well as hemozoin in fibrin and pigmented
456 macrophages (past placental malaria) [47].

457 **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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