Placental Malaria: Histological Findings and Immune Cell Infiltrates in Submicroscopic Infections

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Abstract

Most research on placental malaria is focused on microscopic infection by *Plasmodium falciparum*; there are very few studies on submicroscopic infection. This study aimed to assess alterations of placental tissue associated with placental malaria, to describe the immune cell populations in the placental tissue, and to explore the relationships between the histopathological changes and cell infiltrates. A descriptive, prospective and cross-sectional study was carried out. Women were recruited at hospital obstetric facilities in three municipalities in Northwest Colombia. The histopathological analysis was performed in a total of 132 placentas including 66 placentas with submicroscopic plasmodial infection and 66 that were negatives. Immunohistochemistry was performed on a subset of 75 placentas to determine the distribution of immune cells. Based on histology, there were more immune cells in placentas with submicroscopic plasmodial infection compared with those without infection. The quantity of syncytial knots and calcifications was greater with submicroscopic plasmodial infection, but the quantity of abruption and thrombi was greater in placentas without infection. By immunohistochemistry, we observed a significant increase of CD56+ and CD68+ cells in the infected placentas. Submicroscopic plasmodial infection in the placenta causes tissue alterations and increased immune cell infiltrates. Submicroscopic plasmodial infection is very common in Colombia and can represent a serious threat to mothers and newborns.

Background

Malaria in pregnancy is a significant cause of maternal and infant morbidity. [1–8] When parasites infect the placenta it is known as placental malaria (PM). [9] Most studies have focused on PM diagnosed by thick blood smear (microscopic infection), but submicroscopic infections also cause PM and have pathological consequences for the mother and the fetus. [8, 10–12] In addition, most of the pathology associated with malaria in pregnancy has been observed in *Plasmodium falciparum* infections, but malaria in pregnancy caused by *Plasmodium vivax* can also lead to adverse obstetric outcomes. [8, 13, 14]

The objectives of this study were: 1) to describe the placental histological changes detected in placental submicroscopic plasmodial infections; and 2) to describe the type, quantity and location of immune cell populations in the placental tissue.

The invasion of the endometrium by the trophoblast is a highly controlled process, and that control largely depends on maternal immune cells and their secreted products within the decidual microenvironment. Decidual leukocytes play an important role in this process; they increase in number during the secretory intermediate phase near the time of implantation, and they continue increasing during early pregnancy. [15] There are four main leukocyte populations in the placenta: T cells, decidual natural killer cells, placental macrophages or Hofbauer cell, and dendritic cells. Of the total decidual leukocytes, 70% are decidual natural killer cells, 20–25% are Hofbauer cell, and 1.7% are dendritic cells.
A decreased number of Hofbauer cell, decidual natural killer cells, and dendritic cells have deleterious effects on placental development and on decidual implantation or formation. PM is associated with a number of changes in the placental tissue that are observed by histopathology. Inflammatory cell infiltration in the intervillous space (IVS) is frequently observed during an active placent al malarial infection, primarily from mononuclear cells, which cause inflammation in the IVS as well as in the villi.

In pregnant women, cell-mediated immunity is modulated to promote the development of the placenta and its implantation in the maternal endometrium. But this halt in cell-mediated immunity makes pregnant women more susceptible to intracellular pathogens. However, in malaria-infected pregnant women, an increase in cell-mediated immunity has been observed in the local placental environment with elevated levels of pro-inflammatory cytokines, such as IFN-γ, IL-2, and TNF-α. These cytokine levels correlate with increased Hofbauer cell density, parasite density, and malaria pigments in the placenta. Maternal macrophages are the predominant source of chemokines in the placenta, but fetal cells can also contribute. The chemokines help to recruit macrophages, cytotoxic T cells, B cells, and granulocytes in the placenta and contribute to the pathologies of placental malaria. There is excessive sequestration of parasitized red blood cells and leukocytes in the IVS of placenta and formation of perivillous fibrin clots during malarial infection, which interfere with blood flow across the placenta thus restricting nutrients to the fetus.

Submicroscopic infections are common in pregnant women. Submicroscopic infections can be considered chronic because they are not treated, and the persistence of antigenic stimuli can generate changes in cytokine environments and normal cell populations in peripheral and placental maternal blood. In Colombia, an area of low transmission of both P. falciparum and P. vivax, there is a high frequency of submicroscopic gestational and placental infections, 49% and 57%, respectively. The impact of submicroscopic infections of both species in pregnancy in this area is being explored, and it has been shown that submicroscopic infection during pregnancy is frequent and can affect the health of women and their offspring.

**Methods**

The objective of this study was to describe the placental histological changes detected in placental submicroscopic plasmodial infections.

**Study location**

Women were recruited during 2009–2014 at the hospital obstetric facilities in the municipalities of Monteria (08°45’N, 75°53’W), Puerto Libertador (07°53´35´´N, 75°40´16´´W) and Tierralta (8°10′22″N 76°03′34″O) in the Cordoba department, located in the malaria region of Colombia termed Uraba-Sinu-San Jorge-Bajo Cauca region. This region is homogeneous in terms of ecoepidemiology and malaria transmission. Transmission intensity is low and stable, with no marked fluctuations in the number of
malaria cases during the year. This region has an estimated area of 43,506 km², malaria at-risk population of 2.5 million with a mean annual parasite index of 35.8 cases/1000 inhabitants. Most municipalities in the region are highly endemic for malaria all year. [36] The city of Monteria, the capital of the department of Córdoba, has no malaria transmission; however, due to administrative reasons, some pregnant women residing in Puerto Libertador and Tierralta are referred to Monteria for labor and delivery care.

In the study area, the antenatal care program is free, attended by trained nursing staff, and the appointments are monthly or biweekly after 36 weeks of gestation. At each antenatal care appointment, a thick blood smear (TBS) is taken from the pregnant woman, and she is febrile, the patient is referred to the malaria center for microscopic diagnosis and malaria treatment if necessary. The standard treatment is chloroquine for \textit{P. vivax} malaria and artemether-lumefantrine for \textit{P. falciparum} malaria.

**Sample design and size**

A descriptive, prospective and transversal design was used [37]. The sample was selected for convenience.

The women studied here participated in different studies, all of which included the examination of the blood of the women and placentas at the time of delivery using TBS and qPCR, as well as obtaining samples of placental tissue and clinical and epidemiological information. The goal was to obtain at least 50 placentas with \textit{Plasmodium} submicroscopic placental infection (presence of the parasite detected with qPCR but negative with thick film) and 50 placentas without infection. When this quantity of samples was obtained, the objectives corresponding to what was published in this report were defined.

A total of 136 women and their placentas were included initially, 66 women negative for plasmodial infection during pregnancy and at delivery in both peripheral blood and placental blood (all tests were negatives) and 70 women with placental plasmodial infection. Of the 70 placentas positive by qPCR, four were also positive by TBS, and they were excluded from this study to focus exclusively on submicroscopic placental infection. \textit{P. falciparum} was detected in 54% (36/66) and \textit{P. vivax} in 46% (30/66) of infected placentas.

The placental histopathological study was performed on all placentas, while the immunohistochemistry assays were carried out on a subgroup of 75 placentas: 50 qPCR positives for \textit{Plasmodium} in placental blood, and 25 qPCR negatives.

**Inclusion and exclusion criteria**

Inclusion criteria were voluntary acceptance to participate in the study; permanent residency in the malaria endemic region (> 1 year); negative history of preeclampsia, gestational diabetes, arterial hypertension; and negative HIV and TORCH tests. The exclusion criterion was withdrawal of consent.

**Data and specimen collection**
After inclusion, a clinical-epidemiological questionnaire was completed; in addition, the clinical chart of each woman was consulted. Peripheral maternal blood was taken for the microscopic and molecular diagnosis of gestational malaria. From the placenta, after cleaning with saline, samples of tissue and blood were taken for the diagnosis of PM and the histological and immunohistochemical analysis. For the histological study, two tissue samples were taken, one close to the umbilical cord insertion or the other between the cord insertion and the edge of the placenta. A slide was made from each tissue sample and stained with hematoxylin eosin.

**Malaria diagnosis**

Plasmodial infection was diagnosed by TBS, quantitative real-time PCR (qPCR) and placental tissue histology. Submicroscopic infection was defined as a positive result by qPCR and a negative result by TBS and histology. TBS were read by an experienced microscopist. TBS were defined as negative if 200 fields (100X magnification) were free of parasites [38].

For molecular diagnosis, a hole punch circle (~ 6 mm) from each dried blood spot was used for DNA extraction using the saponin-Chelex method. [39] The qPCR was based on a published method with some modifications. [40] Briefly, the samples were first tested for *Plasmodium* using genus specific primers and a hydrolysis probe (Plasprobe) on the ABI 7500 FAST platform, samples with a cycle threshold < 45 were considered positive for plasmodial infection. The sensitivity of the qPCR assay for the detection of parasite DNA in clinical samples is limited by the input volume in the qPCR reaction, which corresponds to ~ 2.5µL whole blood. Samples considered positive for plasmodial infection were tested in two single species-specific reactions for *P. falciparum* and *P. vivax*, performed using reverse primers specific for each species of plasmodium and combined with the conserved, forward primer, and species-specific probes.

Histological diagnosis was carried out by completely reading the microscopic slides of placental tissue stained with hematoxylin-eosin; the slides were examined by light microscopy under 100X (magnification = 1,000; high-power field) objective lenses. Tissues presenting only parasitized red blood cells (acute PM), or parasitized red blood cells with hemozoin (HZ) (chronic PM) were considered positives. Observing only HZ in placental tissue indicates past infection and not active. [41].

A placenta was considered positive for PM when one or more of the PM diagnostic tests (qPCR, TBS, and histology) had a positive result. Submicroscopic infection was considered when both TBS and histology were negative and qPCR was positive. A placenta was considered negative for PM when all three diagnostic tests were negative.

**Histopathology study**

All placental biopsy specimens were prepared and examined by one of the authors of this report (OMAG), who was supervised by a professional pathologist. Analyses were done without prior knowledge of the maternal characteristics, pregnancy outcomes, or malaria episodes in pregnancy. Details were described
in other reports. [5, 8, 11, 35] Histological slides were examined by light microscopy under 40X (magnification = 400) objective lenses.

Histological variables/events were evaluated quantitatively. This evaluation was done in two ways: a) for these events: abruption, atherosis, necrosis in the decidua, villous edema, villous infarction, hemorrhage in IVS, and thrombus in IVS, the fields with presence of the event were added, and this sum was considered as the amount of each event; b) for these events: fibrin deposits, syncytial knots, chorionic villi, fetal capillaries, capillaries per villa, calcifications in IVS, and immune cells, each event was numerated in each field, and that number was considered as the amount of the event.

Changes or lesions in the placenta were grouped into three broad categories: 1) placental vascular processes (maternal or fetal vascular stromal lesions); 2) inflammatory-immune processes (infectious inflammatory lesions; inflammatory immune / idiopathic lesions); 3) other processes [42]. The variables evaluated were defined considering recent criteria on placental histopathology in general [42–44] and we also included previous criteria on histopathology of placental malaria [5, 6, 13, 21, 45–53]. These definitions are listed in Table 1.
### Table 1
**Definition of histological events**

| Event                   | Description                                                                                                                                                                                                 |
|-------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| **Abruptio**            | Placental abruption hemorrhage; there is accumulation of blood under the decidua and dissection of it. Abruptio represents the rupture of incompletely remodeled spiral arteries due to ischemia-reperfusion or atherosis. When the abruption represents the rupture of maternal veins, usually at the periphery of the placenta, this refers to marginal abruption [42]. |
| **Atherosis**           | Change in the spiral arteries of the decidua, specifically the thickening of the arterial endothelium. The arterioles of the placental bed show signs of fibrinoid necrosis and foam cells. These alterations are placental atherosis and are similar to the changes seen in the vessels of patients with atherosclerosis [70, 71]. |
| **Decidual necrosis**   | Ischemic area with degenerative lesions in the decidua. In decidual necrosis due to infections (malaria, toxoplasmosis, listeriosis, mycoplasmosis, virosis, etc.), focal areas of necrosis with intense infiltration with neutrophils, thrombi inside decidive blood vessels and intense bleeding are common. [45, 46]. |
| **Villous edema**       | Abnormal accumulation of fluid in the stroma of the capillaries of villi, characterized by the expansion or swelling of them and the presence of voids in the stroma. “The accumulation of fluid in the chorionic villus stroma is a poorly elucidated entity. During the second half of pregnancy there are 13% placentas with hairy edema” [72, 73]. |
| **Chorionic villus infarction** | Ischemic necrosis of an organ (tissue death due to lack of blood and subsequently oxygen), usually due to obstruction of the arteries that supply it [43]. Gestational arterial hypertension links the placenta with increased development of infarctions and lower organ weight [73, 74]. |
| **Fibrinoid/fibrinous deposit or fibrin deposit** | Accumulation of fibrin in the stroma of the capillaries of villi or around the capillaries of villi (in the IVS) [43]. The IVS that separate the villi from each other are very variable and can be occupied by an eosinophilic material called fibrinoid [73]. |
| **Syncytial nodules**   | From the second trimester of pregnancy, syncytiotrophoblast cells begin to cluster in compact nests on the surface of the capillaries of villi, leaving spaces devoid of syncytium; those cell clusters are syncytial nodes [75]. The presence of syncytial nodes has a positive correlation with the time and severity of pregnancy hypertension, as well as the presence of fibrin in the capillaries of villi [76]. Exposure to hypoxia and hyperoxia or reactive oxygen species induces the formation of syncytial nodes [45, 77–79]; these phenomena are common in malaria. In term placentas, the prevalence of syncytial nodes in capillaries of villi greater than 33% should be considered increased [73, 80]. |
Abruptio

Placental abruption hemorrhage: there is accumulation of blood under the decidua and dissection of it. Abruptio represents the rupture of incompletely remodeled spiral arteries due to ischemia-reperfusion or atherosis. When the abruption represents the rupture of maternal veins, usually at the periphery of the placenta, this refers to marginal abruption [42].

Chorionic villi

The chorion is the fetal membrane that is in direct contact with the endometrium of the uterus and that covers the chorionic sac. It is formed by syncytiotrophoblast, cytotrophoblast and extraembryonic mesoderm. Capillaries of villi are cytotrophoblast cells that proliferate on the outer surface of the chorionic sac forming cellular clusters that project to the syncytiotrophoblast, and occur at the end of the second week [45]. The various kinds of capillaries of villi differ in caliber, stromal structure, morphology and blood vessel number [73, 81]. The approximate constitution of the term placenta is 40–50% of terminal villi, 25% mature intermediate, 20–25% trunk villi, 5–10% immature intermediate and less than 1% mesenchymal villi [79]. The number of villi seen per high-powered field is significantly increased both in active and treated malaria cases compared to non-malaria controls; there is a significant decrease in villous area in active malaria-infected cases compared to both controls and treated malaria cases [58].

Capillaries

The mesoderm cells in the center of the tertiary capillaries of villi begin to differentiate into small caliber capillaries that form arteriovenous capillary networks that constitute the tertiary capillaries of villi. At the end of the third week, blood begins to circulate through the capillaries of the capillaries of villi [45].

Capillaries per chorionic villus (villous vascularity)

The capillary network of the distal capillaries of villi serves to supply fetal blood after several generations of branching of the vessels that extend from the umbilical cord. The lesions to the integrity of the placental capillary network, which occur in different situations, have consequences that present serious risks to the health of the fetus, the infant and the adult [47, 58, 82, 83].

Hemorrhage in the IVS

Blood flow from the circulatory system, caused by the rupture of blood vessels. In this work, attention was focused on hemorrhages evaluated microscopically, which correspond to “small vessel rupture (fetomaternal hemorrhage)” [42]. “When histology confirms that the bleeding is enclosed by an infarction, the term hematoma should be used” [43].

Thrombus in the IVS

Blood clot as a result of bleeding [73].

Calcifications in the IVS

Deposition of calcium salts in the tissue [73, 84].

Immune cells

Total cells in each zone (decidua, villus and intercellular space). Immune cells have nuclei that are deeply or densely stained (chromatin is large and bulky) and almost fill the cells, with only a slight edge of cytoplasm around the nuclei. This cell can have a nucleus divided into two to five round or ovoid lobes that are connected with thin chains or small chromatin bands. Events associated with immune cells are those of villitis and intervillitis, whose definition or diagnostic criteria are currently highly problematic [15, 18, 24].
In formalin-fixed, paraffin embedded tissues, the EnVision system (Dako) with anti-Human CD4 (Clone 4B12, Dako), anti-Human CD8 (Clone C8/144B, Dako), anti-Human CD14 (Clone TÜK4, Dako), anti-Human CD56 (Clone 123C3), and anti-Human CD68 (Clone PG-M1, Dako) was used and each marker was used on different tissue sections. Briefly, the paraffin sections were deparaffinized and rehydrated in xylene and graded alcohols. After blocking with peroxidase in ChemMate peroxidase-blocking solution (Dako), the slides were incubated with the primary antibodies. After application of the peroxidase-labeled polymer, the slides were incubated with the diaminobenzidine substrate chromogen solution, counterstained with hematoxylin, washed again, dehydrated, and mounted. The immunohistochemical slides were observed using a Zeiss Axio Imager M2 light microscope equipped with a Zeiss Axio Cam HRc Camera to capture images of the placenta. Ten photos were collected per slide, with a 40X objective lens. Subsequently, each photograph was analyzed and the number of cells counted for each photo. The number of positive cells was calculated and analyzed using the Image J software (Image J 1.46r Wayne Rasband National Institutes of Health, USA, https://imagej.nih.gov/ij/index.html).

Statistical analyses

Most of our data were not normally distributed, based on the Kolmogorov–Smirnov test; thus, the non-parametric Mann–Whitney U-test was performed to evaluate differences between the groups. IBM SPSS Statistics (version 24) was used. Comparisons were made between the uninfected placentas group versus the infected placentas group. Significance was accepted for all analyses at P < 0.05. Spearman’s rho was used to measure the correlation between the variables.

Results

A total of 132 women and their placentas were evaluated, 50% (n = 66) with submicroscopic placental plasmodial infection, 50% without infection. *P. falciparum* was detected in 54% (36/66) and *P. vivax* in 46% (30/66) of infected placentas. No statistically significant differences were observed between the study groups in terms of the general characteristics of women (Table 2). However, two clinically important differences were noted: the median hemoglobin level was 1 g/dL lower in the PM group and the median birthweight was 225 g lower in PM.
### Table 2
General characteristics of the pregnant women

| Variable                                  | Placental malaria (PM) |   |   |   |
|-------------------------------------------|------------------------|---|---|---|
|                                           | Without PM (n = 66)    |   |   |   |
|                                           | Without PM (n = 66)    |   |   |   |
| Median IQR 25–75%                         | 22                     | 19–24 | 24 | 19–29 |
| Gestational age at delivery (weeks)       | 38                     | 37–40 | 39 | 38–40 |
| Hemoglobin at delivery (g/dL)             | 11,67                  | 11–12 | 10,83 | 10–12 |
| Birth weight (g)                          | 3100                   | 2900–3400 | 2875 | 2600–3000 |
| Residence time in malarious zone: >5 continuous years | 93%                   | 91% |
| Parity (previous pregnancies):            |                        |   |   |   |
| None (in first pregnancy)                 | 31%                   | 35% |
| One (in 2nd pregnancy)                    | 23%                   | 19% |
| Two (in 3rd pregnancy)                    | 15%                   | 19% |
| With history of malaria in current pregnancy | 25%                   | 26% |

*Non-parametric Mann-Whitney test for two groups. Data source: patient’s medical history.

### Histopathological findings

Some examples of the histopathological findings detected in the infected placentas are presented in Fig. 1. The number of immune cells in the decidua, villi and IVS, as well as the number of syncytial knots and calcifications were significantly higher in infected than in uninfected placentas (Table 3). Necrosis in the decidua was detected exclusively in the presence of PM. Calcifications were abundant in infected placentas but almost absent in the uninfected group. The number of villi and capillaries per villus were reduced in placentas with PM compared with negative placentas. In contrast, edema and abruption were significantly more frequent in uninfected placentas. All histopathological findings were observed in placentas infected by *P. vivax* or *P. falciparum.*
| Variable                      | Placental malaria (PM) | p (M-W) * |
|------------------------------|------------------------|-----------|
|                              | Without PM (n = 66)    | With PM (n = 66) |
|                              | Median | IQR 25–75% | Median | IQR 25–75% |
| Decidua                      |        |            |        |            |
| Atherosis                    | 1      | 0–2        | 0      | 0–1        | 0.226 |
| Necrosis                     | 0      | 0–0        | 0      | 0–0        | 0.804 |
| Abruption                    | 1      | 0–4        | 0      | 0–3        | 0.042 |
| Immune cells                 | 0      | 0–3        | 50     | 35–93      | 0.001 |
| Villus                       |        |            |        |            |
| Infarction                   | 6      | 1–16       | 9      | 1–20       | 0.341 |
| Edema                        | 7      | 1–17       | 5      | 1–12       | 0.029 |
| Syncytial knots              | 86     | 67–119     | 121    | 79–159     | 0.023 |
| Fibrin deposits              | 70     | 54–84      | 70     | 57–93      | 0.764 |
| Villus                       | 358    | 304–403    | 346    | 290–380    | 0.442 |
| Capillaries by villi         | 8      | 6–9        | 8      | 5–9        | 0.589 |
| Immune cells                 | 20     | 9–30       | 35     | 24–51      | 0.039 |
| IVS                          |        |            |        |            |
| Hemorrhage                   | 18     | 9–22       | 11     | 5–18       | 0.639 |
| Thrombus                     | 1      | 0–2        | 0      | 0–1        | 0.838 |
| Calcifications               | 2      | 0–3        | 3      | 0–30       | 0.027 |
| Immune cells                 | 65     | 40–78      | 118    | 82–173     | 0.001 |

* Non-parametric Mann-Whitney test for two groups.

In general, the histopathological studies consider the presence of infected erythrocytes and HZ to classify PM as an acute, chronic or past infection. [51] According to that classification, the 16 cases of submicroscopic PM detected by histopathology were all past infections. The amount of HZ ranged
between 6 to 25 deposits. In the majority of the placentas (70%, 11/16), the HZ was free or phagocytized both in the IVS and in the villus. As expected, no HZ was detected in the villus or IVS in placentas that were qPCR negative.

**Immune cell populations in the placenta**

All immune cell populations increased in the presence of PM (Table 4, with representative images in Fig. 2), but the greatest increases were for CD68+ cells, followed by CD14+ and CD56+ cells, and finally CD4+ and CD8+ cells. Specifically, the increase in cell populations in infected tissue compared to healthy tissue were 98% for CD68+, 78% for CD14+, 34% for CD4+, 31% for CD56+, and 27% for CD8+. CD56+ cells, CD4+, and CD8+ were always observed in the decidua and IVS, while CD68+ cells were also found within the villous stroma in some cases of PM. The staining used for the immunohistochemical study limited the specific determination of the area (decidua, IVS and villi) where the cells were quantified; therefore, the location of the identified cells is indicated in a qualitative way.

### Table 4

| Variable | Placental malaria (PM) | | P (M-W) * |
|----------|------------------------|---|---------|
|          | Without PM (n = 25)    | With PM (n = 50) | |
|          | Median | IQR 25–75% | Median | IQR 25–75% | |
| CD4+ Cells | 11 | 10–12 | 15 | 14–16 | 0.0001 |
| CD8+ Cells | 10 | 9–11 | 13 | 11–15 | |
| CD14+ Cells | 32 | 28–34 | 53 | 51–57 | |
| CD56+ Cells | 78 | 74–80 | 98 | 94–110 | |
| CD68+ Cells | 43 | 30–53 | 91 | 74–98 | |

* Non-parametric Mann-Whitney test for two groups.

**Significant bivariate linear correlations between histological events and immune cells**

Correlation is a statistical technique used to determine the relationship between two or more quantitative variables and indicates (i) the strength of the association; (ii) the direction or meaning of the association; (iii) the shape of the association (indicates the type of line that defines the best fit: straight line, monotonic curve or non-monotonic curve). To analyze the relationship between variables, the “correlation coefficients” were used. The existence of correlation does not imply cause-effect relationships. To explore
the relationship between histological events, between immune cell populations, and between events and cells, a simple linear correlation analysis was performed.

**Uninfected placentas:** 25 significant bivariate linear correlations between histological events and immune cells were found, of which 4 (20%) had $p < 0.05$ and the rest (80%) $p < 0.01$ (Fig. 3). Immune cell infiltrates in decidua correlated with immune cell infiltrates in villi, and the latter correlated with immune cell infiltrates in the IVS.

**Infected placentas:** 24 significant bivariate linear correlations were found between histological events and immune cells, of which 6 (26%) had $p < 0.05$ and 74% had $p < 0.01$ (Fig. 3). Immune cell infiltrates in the IVS correlated with immune infiltrates in decidua and villi, but these two variables did not correlate with each other.

As can be deduced from Fig. 3, the amount and class of correlations identified between histological events has little variation between the two groups.

**Discussion**

An important achievement of this study was to describe the histological alterations that are frequent in placentas with submicroscopic infections. There are reports that do not show associations between submicroscopic infections and negative pregnancy outcomes [10, 32, 54–56], but there are also reports that submicroscopic infection during pregnancy affects the health of the mother and the fetus [10–12, 32, 55, 57–59]. These infections in maternal peripheral blood or in the placenta may cause pathological effects on maternal and infant health, although, apparently, with lesser magnitude than microscopic infections. Considerations of other variables such as the frequency, density, and timing of parasitemia of gestational malaria are also important risk factors for PM. [60]

Here we showed the effects of submicroscopic infection on histopathological changes associated with inflammatory processes, consistent with our previous studies. [5, 11] The placentas with submicroscopic infection had more immune cells and more tissue changes or alterations than the uninfected placentas, except for atherosis, abruption, edema, hemorrhage, and thrombus. These findings confirm the pathogenic nature of the parasite in this organ.

PM is manifested by important changes in placental architecture, such as the presence of inflammatory cell infiltrates, as well as for the release of proinflammatory mediators in the IVS, fibrin deposits, cytotrophoblast cell proliferation, necrosis, and thickening of the trophoblastic basal membrane. [2, 13, 21] In addition, infected erythrocytes, and free or phagocytosed HZ deposits have been observed. [48] In our study, we found similar alterations suggesting that inflammation is generated by the infection leading to the accumulation of proinflammatory immune cells in the decidua and in the IVS. In particular, we noted an increase in NK cells, which could generate a proinflammatory and modulating environment at the expense of the activity of these cells to increase the migration of other cells and the elimination of the parasite. In *P. falciparum* infection, NK cells are known to mediate an important host response as these
cells produce IFN-γ and TNF that contribute to suppress the infection or to develop complications [61]. We also observed a predominance of CD68 + cells, consistent with the proinflammatory environment. These cells respond to the infection by phagocytosing infected erythrocytes and the products of the parasite (HZ and microvesicles). In turn, the increased infiltrate of macrophages and infected erythrocytes, inflammatory reactions, and intervillitis may reduce the exchange of nutrients and oxygen through the placenta, reducing the availability of oxygen to the fetus, and resulting in placental hypoxia and intrauterine growth restriction. [7, 42, 62–64]

The observed increase in immune cells could be explained as a consequence of the production of microvesicles from circulating infected erythrocytes, which come from the maternal and placental circulation. In murine models and in cell cultures, these microvesicles have powerful immunomodulatory activity on monocytes, macrophages, and neutrophils. [65] HZ can also have an immunostimulatory effect on the syncytiotrophoblast, mediated by chemokines that facilitate the recruitment of peripheral blood mononuclear cells to the placenta. [66, 67]

We further explored correlations between different parameters of PM to advance our understanding of the histological changes and the physiological processes that occur during PM. The results of bivariate linear correlations indicated that immune cell infiltrates are the central points of correlation to the histological changes, in both infected and uninfected placentas, while the different cells identified by immunohistochemistry did not participate in interactions with histological events, except CD4 + cells. These correlated events are, essentially, the same in the absence or in the presence of infection.

**Conclusions**

These results demonstrate that the diagnosis of PM by histology lacks sensitivity to detect submicroscopic infection and the frequency of PM in this region is likely to be underestimated. Additionally, it is evident that submicroscopic infection in the placenta generates important changes in its morphology that can affect the development of pregnancy [13, 52, 68, 69]. These findings may affect intervention strategies to reduce the burden of PM in Colombia and in other parts of the Americas, where a microscopic diagnosis of placental infection is essential to administer adequate treatment and avoid complications associated with gestational malaria.

**Abbreviations**

**CD**: cluster of differentiation. **Ct**: cycle threshold. **DNA**: deoxyribonucleic acid. **EDTA**: ethylenediaminetetraacetic acid. **IVS**: intervillous space. **PM**: placental malaria. **qPCR**: quantitative real-time polymerase chain reaction. **TBS**: thick blood smear. **TORCH**: acronym is used universally to refer to a fetus or newborn presenting clinical features compatible with a vertically acquired infection and allows a rational diagnostic and therapeutic approach. The microorganisms classically included are *Toxoplasma gondii*, rubella virus, cytomegalovirus, herpes simplex virus and other agents. **SPI**: submicroscopic plasmodial infection.
Declarations

Ethics approval and consent to participate

The study protocol was reviewed and approved by: a) Ethics Committee of the Sede de Investigaciones Universitarias SIU, Universidad de Antioquia (Medellín, Colombia) (act number 07-32-126: project code 111540820495, contract: 238–2007, Colciencias); b) Ethics Committee of the Instituto de Investigaciones Médicas, Universidad de Antioquia (act number 12: project code 111549326134, contract 611-2009, Colciencias). Each participant gave full informed consent according to the Helsinki convention and the Colombian regulations for this type of research. Each subject voluntarily agreed to participate in the study.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

OMAG, JC-F and EAF conceived the project and designed the experiments, supervised overall design and development and wrote the manuscript. SKY edited the manuscript.

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