Plasmodium falciparum infection during pregnancy impairs fetal head growth: prospective and populational-based retrospective studies

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ABSTRACT

Background: Malaria in pregnancy is associated with adverse effects on the fetus and newborns. However, the outcome on a newborn's head circumference (HC) is still unclear. Here, we show the relation of malaria during pregnancy with fetal head growth.

Methods: Clinical and anthropometric data were collected from babies in two cohort studies of malaria-infected and non-infected pregnant women, in the Brazilian Amazon. One enrolled prospectively (PCS, Jan. 2013 to April 2015) through volunteer sampling, and followed until delivery, 600 malaria-infected and non-infected pregnant women. The other assembled retrospectively (RCS, Jan. 2012 to Dec. 2013) clinical and malaria data from 4697 pregnant women selected through population-based sampling. The effects of malaria during pregnancy in the newborns were assessed using a multivariate logistic regression. According with World Health Organization guidelines babies were classified in small head (HC < 1 SD below the median) and microcephaly (HC < 2 SD below the median) using international HC standards.

Results: Analysis of 251 (PCS) and 232 (RCS) malaria-infected, and 158 (PCS) and 3650 (RCS) non-infected women with clinical data and anthropometric measures of their babies was performed. Among the newborns, 70 (17.1%) in the PCS and 934 (24.1%) in the RCS presented with a small head (SH). Of these, 15 (3.7%) and 161 (4.2%), respectively, showed microcephaly (MC). The prevalence of newborns with a SH (30.7% in PCS and 36.6% in RCS) and MC (8.1% in PCS and 7.3% in RCS) was higher among babies born from women infected with *Plasmodium falciparum* during pregnancy. Multivariate logistic regression analyses revealed that *P. falciparum* infection during pregnancy represents a significant increased odds for the occurrence of a SH in newborns (PCS: OR 3.15, 95% CI 1.52-6.53, p=0.002; RCS: OR 1.91, 95% CI 1.21-3.04, p=0.006). Similarly, there is an increased odds of MC in babies born from mothers that were *P. falciparum*-infected (PCS: OR 5.09, 95% CI 1.12-23.17, p=0.035). Moreover, characterization of placental pathology corroborates the association analysis, particularly through the occurrence of more syncytial nuclear

aggregates and inflammatory infiltrates in placentas from babies with the reduced head circumference.

Conclusions: This work indicates that falciparum-malaria during pregnancy presents an increased likelihood of occurring reduction of head circumference in newborns, which is associated with placental malaria.

Trial Registration: registered as RBR-3yrqfq in the Brazilian Clinical Trials Registry

BACKGROUND

Malaria remains a major global health problem, with approximately one billion people living at high-risk of being infected [1]. *Plasmodium* spp. infection impacts the health of the poorest and marginalized communities in the endemic countries, particularly in infants and pregnant women, with around 125 million pregnancies at risk of infection each year [2]. Malaria during pregnancy, especially falciparum-malaria, can be devastating and fulminant, leading to high mortality for both mother and fetus [3]. During pregnancy, the infected erythrocytes accumulate and sequester in the placental intervillous space, causing placental histopathological changes, which triggers an exacerbated inflammatory response that is highly detrimental [4]. The deleterious effects caused by malaria infection during pregnancy depend on various factors, such as the woman's immunity, the number of previous pregnancies and the trimester of gestation, with primigravida and secundigravida women most susceptible and suffering the greatest consequences [5].

A heightened inflammatory response perturbs the maternal-fetal interface and impairs critical placental functions. Therefore, maternal malaria presents a major impact on fetus and newborns, being the main cause of abortion, stillbirth, premature delivery and fetal death in malaria-endemic countries [3]. Low birth weight (LBW) caused by prematurity or intrauterine growth retardation (IUGR) is commonly observed in babies born from mothers who had malaria during pregnancy, contributing to around 100,000 infant deaths each year [3,5,6]. Additionally, *in utero* exposure to malaria parasites has been shown to impact the fetus or newborn head circumference (HC), a proportional reduction as an outcome of the IUGR [7,8]. Albeit, no further studies have tried to unpick a specific disproportionate HC reduction associated with malaria during pregnancy.

Several studies have reported the association of intrauterine infections with a high risk of the newborn to have LBW and brain injury [9]. A group of microorganisms designated as TORCH, an abbreviation for *Toxoplasma*, rubella, cytomegalovirus, and Herpes simplex that now also comprise *T. pallidum* (Syphilis), hepatitis virus, and HIV, and recently, the Zika virus are frequently associated with reduced HC in newborns [10,11]. The more adverse consequence that results from

these infections is microcephaly at birth, which is defined by a reduction of the occipitofrontal HC of more than two standard deviations (SD) below the median compared to age and sex-matched control population [12]. Although the brain insult is defined by the cranium size, it also reflects a reduction of the brain volume and an impairment of cognitive abilities [12].

Thus, to investigate the relation of malaria during pregnancy on the fetus head growth, we analyzed data from a prospective and a retrospective cohort from newborns delivered between 2012 and 2015 in Cruzeiro do Sul (Acre State in the Southwestern Brazilian Amazon Basin), where 46% of the total falciparum-malaria Brazilian cases occur [13,14].

METHODS

Setting

Two cohort studies were conducted in the Amazonian region of the "Alto do Juruá" valley (Acre, Brazil), evaluating maternal-child pairs data of births at the general maternity ward, Hospital da Mulher e da Criança do Juruá (HMCJ, Cruzeiro do Sul), where approximately 90% of the total deliveries in the region occur. "Alto do Juruá" valley is in the extreme southwest of the Brazilian Amazon Basin, covering an area of 74,965 km^2, predominantly rainforest, and a population of ~200,000 inhabitants. It is limited to the north by the Amazonas state, to the east by the Acre Valley (Acre), and to the south and west by Peru (Fig. 1). This is a region of high malaria endemicity in Brazil, with an annual parasite incidence above 100, where *P. vivax* is responsible for 70-80% of the malaria cases, and where 46% of the total *P. falciparum* Brazilian cases occur [14,15]. In this region, 18% of women acquire *Plasmodium* infection during pregnancy [13].

Prospective cohort study (PCS)

- **Design and participants**

A total of 600 pregnant women were enrolled through volunteer sampling of equal numbers of *P. falciparum*-, *P. vivax*-infected, and non-infected pregnant women, and followed until delivery, between January 2013 and April 2015. The women were recruited during their first pregnancy visit to the antenatal care (ANC) clinic. Each pregnant woman was followed by a trained nurse, which involved at least two domiciliary visits, at the second and third trimester, to monitor their clinical state, in addition to the usual prenatal care in health care services.

- **Samples collection**

At the time of recruitment, data was collected on socioeconomic, clinical, and obstetric variables, and peripheral blood and thick and thin blood smears were used to diagnose and confirm malaria infection. During the domiciliary visits, clinical and obstetric data were obtained, and collected a peripheral blood sample. An additional blood sample was collected in each episode of malaria

during pregnancy. At the time of delivery, clinical data were collected from mother and newborn, as well as a placental biopsy and blood samples.

- **Samples processing**

The peripheral and placental blood was collected in heparin tubes and then separated into plasma and whole blood cells using a centrifuge. Thin and thick blood smears were stained with Giemsa. The placental biopsies were fixed in 10% neutral buffered formalin at 4°C until they could be sent to the University of São Paulo for processing. Paraffin-embedded 5µm sections of placental tissue were stained with Hematoxylin-Eosin (H&E) or Giemsa for histological examination. Total DNA was obtained from whole blood cells using a commercially available extraction kit (QIAmp DNA Mini Kit, Qiagen), following the manufacturer's instructions.

- **Gestational age estimation**

The gestational age of all women from the PCS was estimated by woman's last menstrual period (LMP) and adjusted by ultrasound during the first trimester of pregnancy.

- **Newborns classification according to head circumference**

Based on the gestational age, and on the HC size and gender, each newborn from the PCS was assigned into groups using the INTERGROWTH-21st Project [16]. An individual was in a normal head circumference (NHC) range if their HC was within one SD of the median. Newborns with HC below one SD below the median were considered to have a small head (SH) [17]. Newborns with HC below two SD below the median were classified as having microcephaly (MC) [12].

- **Screening of malaria infection**

Malaria during pregnancy was diagnosed from thin and thick blood smears by two experts in microscopy of the endemic surveillance team of Cruzeiro do Sul (Acre, Brazil). Furthermore, all samples collected throughout the pregnancy were screened for the presence of malaria parasites, by microscopy and confirmed by a real-time PCR technique (PET-PCR). This technique detects in multiplex the *Plasmodium* spp. and *P. falciparum,* and in singleplex *P. vivax* if only *Plasmodium* spp. is detected in the first PCR. PET-PCR has a detection limit of 3.2 parasites/ml [18]. The real-

time PCR was performed on the 7500 Fast Real-Time PCR System (Applied Biosystems, ThermoFisher). All the women who had malaria during pregnancy were treated with antimalarial drugs under medical prescription, according to the Brazilian Ministry of Health (MoH) guidelines, with further treatment confirmation.

- **Histopathology evaluation**

The histopathologic examination involved using placental tissue slides. The Hematoxylin-Eosin-staining allowed evaluating the syncytial nuclear aggregates (SNA), fibrinoid necrosis, and fibrin deposition [19]. The hemozoin presence was assessed through microscopy of polarized light [20]. The leukocyte (CD45) and monocyte inflammatory infiltrate (CD68), and the villous vascularity (CD31) have been evaluated by immunohistochemistry using the tissue microarray (TMA) technique, conducted at the AC Camargo Hospital, in São Paulo, Brazil, as described elsewhere [21,22]. The proliferation index was calculated through quantitative image analysis of anti-Ki-67/DAB staining [23]. Supplementary Table S9 describes these procedures in detail. The images of placenta were captured by a Zeiss Axio Imager M2 light microscope equipped with a Zeiss Axio Cam HRc camera and analyzed by Image J software (http://imagej.nih.gov/ij).

- **Angiogenic factors and Leptin measurement**

The angiogenic factors, vascular endothelial growth factor A (VEGFA, and it receptors VEGFR1/FLT1 and VEGFR2/FLK1), angiopoietins 1 and 2 (ANG-1 and ANG-2, and their associated soluble receptor the TEK receptor tyrosine kinase (TIE-2)), and the leptin hormone were measured in placental plasma (1:20 dilution for all factors) using the DuoSet ELISA development kits (R&D), according to manufacturer's guidelines.

- **Screening of other infectious agents**

All pregnant women were screened in the local ANC clinics for toxoplasmosis, hepatitis, syphilis, and HIV by measuring antibodies titers, following the Brazilian MoH guidelines. Further, peripheral plasma from women that delivered babies with small head and microcephaly, irrespective of the infection status and *Plasmodium* species, was tested to confirm the absence of

other infectious agents during pregnancy. Tests for *Toxoplasma gondii*, Rubella, Cytomegalovirus, Herpes simplex virus, Syphilis, HIV, Dengue virus, Chikungunya virus, and Zika virus were performed retrospectively by ELISA assays in peripheral blood collected until the 28 weeks of gestation. In pregnant women that delivered babies with microcephaly, plasma samples of two different time points of the pregnancy were tested. All the serological tests were performed using commercially available kits: HIV 1/2 and total Syphilis (Symbiosys) and IgG/IgM to Toxoplasmosis, Rubella, Cytomegalovirus and Herpes simplex (TORCH) (Virion\Serion), and used according to the manufacturer's instructions. To detect Dengue, Chikungunya, and Zika current viral infections, qualitative assays were carried out by IgM capture using a specific viral antigen for DENV, ZIKV, and CHIKV, as previously described [24]. The identification of specific IgG antibodies to CHIKV was performed using a specific viral antigen [24], and to DENV and ZIKV were made with an antigen derived from a whole DENV-2 NS1 protein and a portion of the NS1 protein, respectively (unpublished data). Developing color was quantified on an automatic microliter plate reader Spectramax Plus 384 (Molecular Devices). The results were expressed as optical density (OD) at 405/630 nm or 450/630 nm (Virion/Serion and Symbiosys/Alka Kits, respectively). In TORCH analyses, the presence of IgG and IgM antibodies were classified as positive, negative or borderline according to an OD range adopted by standard positive control mean. For Rubella and *Toxoplasma gondii* (IgG) avidity test was performed according to the manufacturer's specifications (Virion/Serion), and in all TORCH IgM tests, we use the rheumatoid factor absorbent reagent (# Z200, Virion/Serion). All the kits followed the validation criteria, and the presence of IgG and IgM antibodies for Syphilis and HIV antigens were determined by comparing the absorbance value of serum samples with the cut-off value of standards of reference controls and classified as positive or negative. All tests were performed without the operator knowledge of the group classification for each sample. If the test was inconclusive the screen was repeated using samples from two different gestational time-points. Newborns were excluded from the analysis whenever their mothers presented antibody titers for IgM.

- **Measurement of cytokines/anaphylatoxins by bead array**

The levels of the cytokines IL-12p70, TNF, IL-10, IL-6, IL-1b, and IL-8 in the placental plasma, were detected and quantified by a CBA human inflammatory kit (BD Biosciences) that was used according to the manufacturer's protocol. For complement activation studies (measuring C3a, C4a, and C5a) the CBA human anaphylatoxin kit (BD Biosciences) was used. The samples were analyzed in a two-laser BD FACSCalibur flow cytometer with CellQuest version 5.2 software (BD Biosciences), and concentrations computed using FCAP array software version 3.0.1 (BD Biosciences). All plasma samples were processed and kept at -80°C in Cruzeiro do Sul until they were sent to the University of São Paulo.

Retrospective cohort study (RCS)

- **Design, participants and data collection**

A total of 4697 maternal-child pairs were selected retrospectively through a population-based sampling of all deliveries occurring between January 2012 and December 2013. The data from the Brazilian Epidemiological Surveillance Information System (SIVEP)-Malaria of the mother malaria infection status during pregnancy was assembled with the clinical and anthropometric data present in the medical records of the mother and the newborn. This was followed by the collection and collation of the data to evaluate the newborns further.

- **Gestational age estimation**

The gestational age in the RCS was established mainly by the woman's last menstrual period (LMP). These data were obtained from the medical records. The LMP method is recommended by the Brazilian MoH for gestational age calculation when it is not possible to use ultrasound.

- **Newborns classification according to head circumference**

Based on the gestational age estimation methodologies, and on the HC size and gender, each newborn from RCS was assigned into groups using the WHO child growth standards (WHO-CGS) [25]. Gestational age assessment is considered accurate when acquired through ultrasound

241 performed early in the first trimester, but the date of the last menstrual period is considered
242 unreliable [26]. According to WHO guidelines, the WHO-CGS provides an appropriate reference
243 standard for term neonates when gestational age is not reliably known. An individual was in a
244 normal head circumference (NHC) range if their HC was within one SD of the median, (boys $33.2 \geq$
245 $HC \leq 35.7$, girls $32.7 \geq HC \leq 35.1$). Newborns with HC below one SD below the median were
246 considered to have a small head (SH) (boys $HC < 33.2$, girls $HC < 32.7$) [17]. Newborns with HC
247 below two SD below the median were classified as having microcephaly (MC) (boys $HC < 31.9$,
248 girls $HC < 31.5$) [12].

249 ▪ **Screening of malaria infection**

250 Malaria during pregnancy was diagnosed from thin and thick blood smears by microscopists of the
251 endemic surveillance team of Cruzeiro do Sul (Acre, Brazil), whenever women show suspicious
252 malaria symptoms. These data were obtained from the Brazilian Epidemiological Surveillance
253 Information System (SIVEP)-Malaria. All the women who had malaria during pregnancy were
254 treated with antimalarial drugs under medical prescription, according to the Brazilian MoH
255 guidelines.

256 ▪ **Screening of other infectious agents**

257 All pregnant women were screened in the local ANC clinics for toxoplasmosis, hepatitis, syphilis,
258 and HIV by measuring antibodies titers, following the Brazilian MoH guidelines.

259

260 **Newborn anthropometric measures**

261 In the two cohort studies, PCS and RCS, the newborn anthropometric measures were obtained
262 immediately after the delivery, maximum within 24h, by trained nurses. Weight was measured in
263 grams (g) using digital pediatric scales, with a precision of 5 g, and the length and occipitofrontal
264 head circumference (HC) were measured in centimeters (cm), using a non-stretching flexible
265 measuring tape. Rohrer's ponderal index is the newborns' weight in grams divided by the cube of
266 the length in centimeters, and babies are considered proportional when values are above 2.5,

corresponding to the 10th percentile [27]. An Apgar score indicates the physical condition of the newborn, relative to its response to stimulation, skin coloration, heart rate, respiratory effort, and muscle tone. If the Apgar Score is between 7 and 10 the newborn is considered normal; if it is between 4 and 6 it is indicative that some assistance for breathing might be required; and below 4, the baby needs several interventions [28].

Exclusion criteria

Our analysis was restricted to babies that had been born at term (37 - 42 weeks of gestation) with at least 2500 grams of weight in a single birth and from mothers of fertile age (13 - 47 years old). Women were excluded if they had a history during pregnancy of smoking, drug use and/or alcohol consumption, and who presented with infections (TORCH, HIV, Hepatitis B virus, Hepatitis C virus, Syphilis, Dengue, Chikungunya and Zika virus), and/or other comorbidities (e.g. hypertension, pre-eclampsia/eclampsia, diabetes mellitus, preterm delivery, stillbirth, and newborn with congenital malformation). Due to the extremely high percentage of C-sections performed in Brazilian maternity units, women who underwent a C-section were not excluded from the study.

Statistical analyses

Data were analyzed using R (r-project.org), Stata (StataCorp), Minitab 18 and GraphPad Prism software. Continuous variables were summarized using means and SD, medians, and interquartile ranges (IQR). Categorical variables were summarized using frequencies and percentages. Differences between groups were evaluated using Mann-Whitney U-tests accordingly. Categorical data and proportions were analyzed using chi-square tests. All *p*-Values were 2-sided, at a significance level of 0.05. To assess the association between malaria and microcephaly, adjusted odds ratios (OR) with 95% confidence intervals (CI) were estimated using a multivariate logistic regression approach. These models included infection by malaria (no/yes), maternal age ($\geq$ 18 years old / $\leq$ 17 years old) and the number of gestations (two or more/one) as explanatory variables and

SH (yes/no) or microcephaly (yes/no) as response variables. The first category for each explanatory variable was considered as reference [29]. Missing data were imputed or "filled in" within a multiple imputation framework using the "MICE" library within the R software [30,31]. In particular, 5 datasets were completed and the results pooled across allowing for the uncertainty in the imputation process.

The current sample sizes present a deviation from those proposed at the outset. It was proposed to enroll ~400 infected and ~800 non-infected pregnant women into the prospective cohort study. We were unable to recruit to this 2:1 ratio, as some initially included in the non-infected group, were transferred to an infected group upon *Plasmodium* molecular detection.

The manuscript was written according to the STROBE statement guidelines.

RESULTS

Study Population

A total of 600 pregnant women were enrolled in a prospective cohort study (PCS) and followed until delivery. Of the first eligible maternal-child pairs, 409 (68.2%) met the inclusion criteria (Fig. 2). Among the 409 newborns, 251 were born from mothers that had malaria infection during pregnancy, *P. vivax* (*Pv*), *P. falciparum* (*Pf*) or both (mixed) (Fig. 2). Overall, there were no relevant maternal and newborns baseline differences between the distinct groups (Supplementary Tables S1 and S2). Nonetheless, women that were *Plasmodium*-infected presented few characteristics at delivery that were slightly different from the Non-Infected group: less weight gain, lower hematocrit, lower hemoglobin, and reduced placental weight (Supplementary Tables S1).

Reduced head circumference in newborns from women infected with *P. falciparum* during pregnancy

The frequency distribution of the newborns HC born from non- (NI) and malaria-infected mothers (Malaria), including LBW and preterm babies, evidenced differences between the two groups. The Malaria group displayed a deviated peak and spread to the left when compared with the NI group, indicative of more newborns with reduced HC ($p = 0.005$) (Fig. 3a). Nevertheless, to assure that the observed difference was not due to the LBW and preterm babies, these newborns were removed from the analysis and segregated the malaria-infected group into *Plasmodium* species infected groups. Even though, it was possible to observe an apparent deviation of the peak of the *P. falciparum*-infected group (*Pf*) from the non-infected (NI) ($p = 0.023$) (Fig. 3b), indicating a higher frequency of babies with smaller HC when mothers are infected by *P. falciparum*.

Among the evaluated newborns in the PCS, 70 (17.1%) babies presented with a small head (SH), including 15 (3.7%) with microcephaly (Fig. 3c). The evaluated babies were considered proportionate through the Rohrer Index, independently of the HC size (Supplementary Table S3). Further, to evaluate the association of malaria during pregnancy with fetus head growth, the

newborns were segregated by HC and the mother infection status: non-infected, *P. vivax*-, mixed- or *P. falciparum*-infected. The prevalence of newborns with SH was higher among babies born from women infected with *P. falciparum* (30.7%) during pregnancy. Similarly, the prevalence of microcephaly doubled when a *P. falciparum* infection has occurred (8.1%) (Fig. 3c). In fact, a multivariate logistic regression analysis identified *P. falciparum* infection as increasing the odds of occurring SH in newborns (OR 3.15, 95% CI 1.52-6.53, $p = 0.002$) (Fig. 3c). Likewise, it revealed a higher likelihood of occurring microcephaly in babies born from mothers that were *P. falciparum*-infected (OR 5.09, 95% CI 1.12-23.17, $p = 0.035$) (Fig. 3c). Strikingly, *P. vivax* infection during pregnancy was not found to be associated with reduced HC (for SH, OR 1.30, 95% CI 0.66-2.59, $p = 0.449$). Maternal-child pairs that presented misleading factors such as TORCH infections, Syphilis, HIV, Dengue, Chikungunya, and Zika virus, and alcoholism and drug use declared in the medical records, or identified in all mothers that delivered babies with were discarded SH (Supplementary Tables S4 and S5).

Reduced head circumference in newborns is associated with placental malaria

Further, several placental parameters were evaluated to ascertain the relation of placental malaria due to *P. falciparum* infection with the SH occurrence. Strikingly, babies with SH (*Pf*-SH) born from mothers that had their first infection later in gestation (median [IQR], 25.5 weeks [18.0-32.5], $p = 0.014$) when compared with NHC (19.0 weeks [12.0-29.3]). Moreover, much of the placental malaria manifestation in newborns with SH (*Pf*-SH) or microcephaly (*Pf*-MC) was due to a past *P. falciparum* infection (54% and 72%, respectively), as opposed to 48% in placentas from newborns with NHC (*Pf*-NHC) (Table 1).

The analysis of placental histology parameters and angiogenic factors disclosed substantial differences between non-infected controls and *P. falciparum*-infected groups. Of note, in all *P. falciparum*-infected groups, we observed higher monocytes infiltrate (median[IQR], *Pf*-NHC 7.0 [5.0-13.0], $p < 0.0001$; *Pf*-SH 9.5 [5.5-15.0], $p < 0.0001$; *Pf*-MC 9.0 [6.0-11.0], $p = 0.018$ vs Non-

Infected 4.0 [2.0-7.0]) (Fig. 4c, d). On the other hand, the syncytial nuclear aggregates (SNA) and Leptin alterations were only observed in infected placentas of babies with SH and MC. Remarkably, SNA that have a long-standing association with placental pathologies [32], presented excessive formation in the *Pf*-SH and *Pf*-MC groups (17.5 [12.0-24.5], $p = 0.002$ and 18.0 [12.0-30.0], $p = 0.023$, respectively) when compared to the Non-Infected (13.0 [10.0-17.0]) (Fig. 4g, h), as well, when *Pf*-SH was compared to *Pf*-NHC. Moreover, the Leptin levels were markedly reduced in the *Pf*-SH and *Pf*-MC groups (19.5 [4.5-37.2], $p = 0.013$ and 16.7 [9.0-26.7], $p = 0.027$, respectively) when compared to the Non-Infected (33.1 [17.2-47.4]) (Fig. 5i). Complete data details can be found in Supplementary Table S6.

Furthermore, evaluation of inflammatory factors in the placental plasma revealed differences mainly between the Non-Infected group and the *Pf*-NHC group. Though, the *Pf*-SH group shows statistically significant higher IL8 and smaller C3a plasma levels (45.1 [22.1-85.9], $p = 0.044$; and, 3.0 [0-5.5], $p = 0.014$, respectively) when compared to the Non-Infected group (25.5 [15.7-52.2], and, 4.5 [3.2-6.6], respectively) (Supplementary Table S7). These results support a placental dysfunction upon *P. falciparum* infection, which in some parameters are specifically heightened in placentas derived from babies with reduced HC, like the syncytial nuclear aggregates.

Retrospective cohort study corroborates the reduced head circumference association with *P. falciparum* infection

Further, a population-based retrospective cohort study (RCS) was conducted to confirm the association results. A total of 4697 maternal-child pairs were included, and upon application of the exclusion criteria, 3882 (83%) newborns remained to be evaluated, of which, 232 were born from mothers that had malaria infection during pregnancy (Fig. 2). Overall, there were no significant differences in baseline characteristics between the PCS and the RCS (Supplementary Tables S1, S2, and S8). The evaluation of the frequency distribution of the newborns HC born from non- (NI) and malaria-infected mothers (Malaria), showed differences between the two groups ($p = 0.008$) (Fig.

382 6a). Identical to the PCS, when the LBW and preterm babies were removed from the analysis, and
383 the malaria-infected group segregated, the *P. falciparum*-infected group (*Pf*) presented a deviated
384 peak from the non-infected (NI) ($p = 0.015$) (Fig. 6b). Indicative of a higher frequency of newborns
385 with reduced HC when mothers are infected with *P. falciparum* during pregnancy.
386 The evaluated newborns included 934 (24.1%) babies with SH and 161 (4.2%) with microcephaly.
387 In the RCS, similarly to the PCS, the prevalence of newborns with SH was more than one-half
388 higher (36.6%) among babies born from *P. falciparum*-infected mothers, and the microcephaly
389 prevalence almost doubled in the presence of a *P. falciparum* infection (7.3%) (Fig. 6c).
390 Analogously, the multivariate logistic regression analysis revealed that *P. falciparum* infection
391 increases the odds of occurring SH in newborns (Odds ratio [OR] 1.91, 95% CI 1.21-3.04, $p =$
392 0.006) (Fig. 6c). Altogether, these results demonstrate that *P. falciparum* infection during
393 pregnancy increases the likelihood of occurring reduced HC in the newborns, corroborating the
394 results obtained in the PCS.

DISCUSSION

It is well-established that malaria during pregnancy increases the risk of adverse fetal outcomes, such as abortion, IUGR, premature births and LBW. We show for the first-time evidence that *P. falciparum* infection during pregnancy is significantly associated with the occurrence of reduced HC in the newborns, and to some extent, with microcephaly. The revealed newborn HC reduction is independent of the already known impact that malaria has on the whole fetal growth, as LBW and preterm newborns were deliberately excluded from our analysis.

The increased risk for developing reduced HC associated with *P. falciparum* infection was supported by a prospective study (PCS) (Odds Ratio (OR) 3.15, $p = 0.002$) and subsequently corroborated by a retrospective study (RCS) (OR 1.91, $p = 0.006$). Remarkably, in the prospective study, the OR doubles when we consider only the microcephaly cases (OR 5.09, $p = 0.035$). These observations reinforce the knowledge that malaria during pregnancy increases the risk of problems in fetal development [3–5].

We hypothesize that the placental inflammatory process acting upon *P. falciparum* infection is contributing to impair the fetal head growth. This hypothesis is supported by the observation of histopathological alterations, combined with an imbalance in angiogenic factors production and inflammatory factors in placentas from babies with congenital SH or microcephaly when mothers were *P. falciparum*-infected. A local inflammation can generate a frame of hypoxia/ischemia that would alter the transportation of both nutrients and respiratory gases to the unborn baby, which can impact on cranial malformation due to the lack of an adequate supply of nutrients and oxygen [33]. Also, the oxidative stress caused by hypoxia leads to several structural and functional alterations in the intrauterine development [34]. This scenario is often observed in cases of placental malfunction due to different etiologies, and prolonged and premature labor [35].

Interestingly, the values of SNA or syncytial knotting, which has been associated with IUGR due to local hypoxia/oxidative stress [32], were highly increased in placentas from the *Pf*-SH and *Pf*-MC groups when compared to the other control groups. Syncytial knotting has repeatedly been observed

421 in placentas from *P. falciparum*-exposed women [4,19,36]. In fact, the major placental alterations
422 observed, including syncytial knots and monocytes inflammatory infiltrate, are consistent with
423 previous reports on placental inflammatory responses due to sequestration of *P. falciparum*
424 parasites in the placenta, which characterizes the placental malaria development [4,5,19]. The
425 evaluation of cytokine levels and complement in our samples did not show an overall alteration.
426 Nevertheless, these only reflect a picture at the moment of birth. It is unsurprising that *P. vivax*
427 infection was not associated with the head reduction phenotype, as this parasite is known as not
428 sequestering in the placenta. Previous studies have demonstrated that *P. vivax* infection during
429 pregnancy induces a less placental inflammatory process when compared with *P. falciparum*
430 infection [19].
431 The presence of residual tissue lesions and impaired leptin production constitute clear evidence of
432 damage. In fact, the *Pf*-SH and *Pf*-MC groups presented deregulated leptin levels. The impaired
433 production of leptin, a hormone commonly produced in substantial amounts by the placenta, can be
434 related to placental inflammation upon infection. Also, leptin has been shown associated with fetal
435 growth restriction [37]. Regarding the *Pf*-SH group, few observed differences reached statistical
436 significance, possibly due to the small sample size of this group, but the overall placental malaria
437 phenotype is more prominent and widespread than in non-infected and *Pf*-NHC groups.
438 Nevertheless, it is unclear how placental alterations due to inflammation impact on the development
439 of the fetus.
440 Currently, much of what is known about falciparum gestational malaria is based on studies
441 performed in African high transmission areas, which in general are settings that have precarious
442 health systems and inadequate or late treatment provision. In Brazil, approximately 85% of the
443 infections are caused by *P. vivax. P. falciparum* is only transmitted in specific regions, including in
444 the one evaluated in this work (“Alto do Juruá” valley, Acre), where it is responsible for 46% of the
445 total infections in Brazil [13,14]. Interestingly, despite Brazil being a low transmission area for

446 malaria with effective control strategies and early treatment provision, we observed adverse events
447 in newborns similar to those reported in areas of high endemicity.
448 Surprisingly, the prevalence of microcephaly (HC < -2 SD) observed by us is far higher than what
449 has been previously reported by the Brazilian Ministry of Health [12]. Two independent studies
450 have recently evaluated retrospectively babies born in two different Brazilian regions, and also
451 reported a higher prevalence of microcephaly in babies born before the Zika outbreak [38,39]. In
452 one, 16,208 infants born between 2012 and 2015 in the Paraiba State (Brazil) were evaluated, and
453 4.2 to 8.2% of microcephaly prevalence was reported, depending on the classification criteria [38].
454 In the other, 8,275 babies born between 2011 and 2015 in the southeastern and mid-western
455 Brazilian region were evaluated, and an overall prevalence of microcephaly of 5.6% was identified
456 [39]. In fact, it is puzzling that a country like the USA with about 3.5 millions of births per year
457 reports annually approximately 25,000 infants with microcephaly [40]; on the other hand, Brazil
458 with about 3 million births per year reported around 150 microcephaly cases annually, before Zika
459 epidemy [41]. These observations indicate an inconsistency of the data released by the Brazilian
460 authorities probably due to under-reporting.
461 Our work has some potential limitations. First, the babies' HC was only assessed at birth, since it
462 was not possible to perform the morphometric measures through ultrasonography during pregnancy
463 in the public health system, as well as the possibility of acquiring newborn head imaging. Second,
464 reduction of HC has different etiologies, namely, genetic causes and action of infectious agents.
465 While we have discarded misleading factors, such as TORCH infections, Syphilis, HIV, Dengue,
466 Chikungunya and Zika virus, as well as smoking, alcoholism and drug use, studies to detect genetic
467 abnormalities in those patients were not performed. Third, although in both the PCS and the RCS
468 the logistic-regression analysis indicates a strong association between SH and *P. falciparum*
469 infection, we only had access to few placentas. The smaller sample size has limited the statistical
470 analysis; however, most of the parameters analyzed indicated intensified placental malaria when
471 compared to placentas from newborns with normal head size.

CONCLUSION

This work provides evidence that *P. falciparum* infection during pregnancy can impact the head growth of the fetus, which leads to small heads and in extreme cases to microcephaly. If our results are confirmed, the consequences of gestational malaria over fetal neurological development, which can lead to poor neurocognitive and behavioral development, represents a serious long-term health problem. Physicians should periodically assess the development and academic achievements of these children, with a comprehensive neurocognitive evaluation, to guide preventive and rehabilitative assistance that might improve outcomes. Extensive epidemiological prospective studies, involving the collection of biological, clinical, and socioeconomic data and potential confounding factors, are required to establish the prevalence of SH and microcephaly and its association with malaria. Our work reinforces the urgent need to protect the pregnant women and their unborn babies from the devastating effects of malaria infection.

ABBREVIATIONS

ANC: Antenatal care; **ANG-1 and ANG-2:** Angiopoietins 1 and 2; **CI:** Confidence intervals; **cm:** centimeters; **g:** Grams; **HC:** head circumference; **H&E:** Hematoxylin-Eosin; **HMCJ:** Hospital da Mulher e da Criança do Juruá; **IUGR:** Intrauterine growth retardation; **IQR:** Interquartile ranges; **LBW:** Low birth weight; **LMP:** Last menstrual period; **MC:** Microcephaly; **MoH:** Ministry of Health; **NHC:** Normal head circumference; **NI:** Non-infected; **OD:** Optical density; **OR:** Odds ratio; **PCS:** Prospective cohort study; **Pf:** *Plasmodium falciparum*; **Pv:** *Plasmodium vivax*; **RCS:** Retrospective cohort study; **SD:** standard deviations; **SH:** Small head; **SIVEP:** Epidemiological Surveillance Information System; **SNA:** Syncytial nuclear aggregates; **TIE-2:** TEK receptor tyrosine kinase; **TMA:** Tissue microarray; **TORCH:** abbreviation for Toxoplasma, rubella, cytomegalovirus, and Herpes simplex; **VEGFA:** Vascular endothelial growth factor A; **WHO:** World Health Organization; **WHO-CGS:** WHO child growth standards.

DECLARATIONS

Ethics approval and consent to participate

Ethical clearance was provided by the committees for research of the University of São Paulo and the Federal University of Acre (Plataforma Brasil, CAAE: 03930812.8.0000.5467 and 03930812.8.3001.5010, respectively), according to Resolution nº 196/96 of Brazilian National Health Committee. All the study participants or their legal guardians (if minors) gave written informed consent. The authors have agreed to maintain the confidentiality of the data collected from the medical records and databases, by signing the Term of Commitment for the Use of Data from Medical Records. The study was conducted in accordance with the Declaration of Helsinki and is registered in the Brazilian Clinical Trials Registry as RBR-3yrqfq.

Consent for publication

Not applicable.

Availability of data and materials

All relevant data are available from the authors on request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JGD, RMS, SE, and CRFM designed the study. JGD, RMS, FAL, CLB, OM, DSC, EPMP, MPC, PMAZ, MAGG, SE, LAG, and CRFM were involved in data acquisition and scientific input. JGD, RMS, FAL, CLB, OM, DSC, EPMP, MPC, PMAZ, EB, MAGG, SC, TGC, SE, LAG, and CRFM contributed to the analysis and/or interpretation of data. ACPL, JMS, and TGC performed the multivariate logistic regression analysis. LAG and CRFM wrote the manuscript and compiled the information in the Supplementary information. CRFM and SE were the main funders of this work.

CRFM have had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors reviewed and approved the final version of this manuscript.

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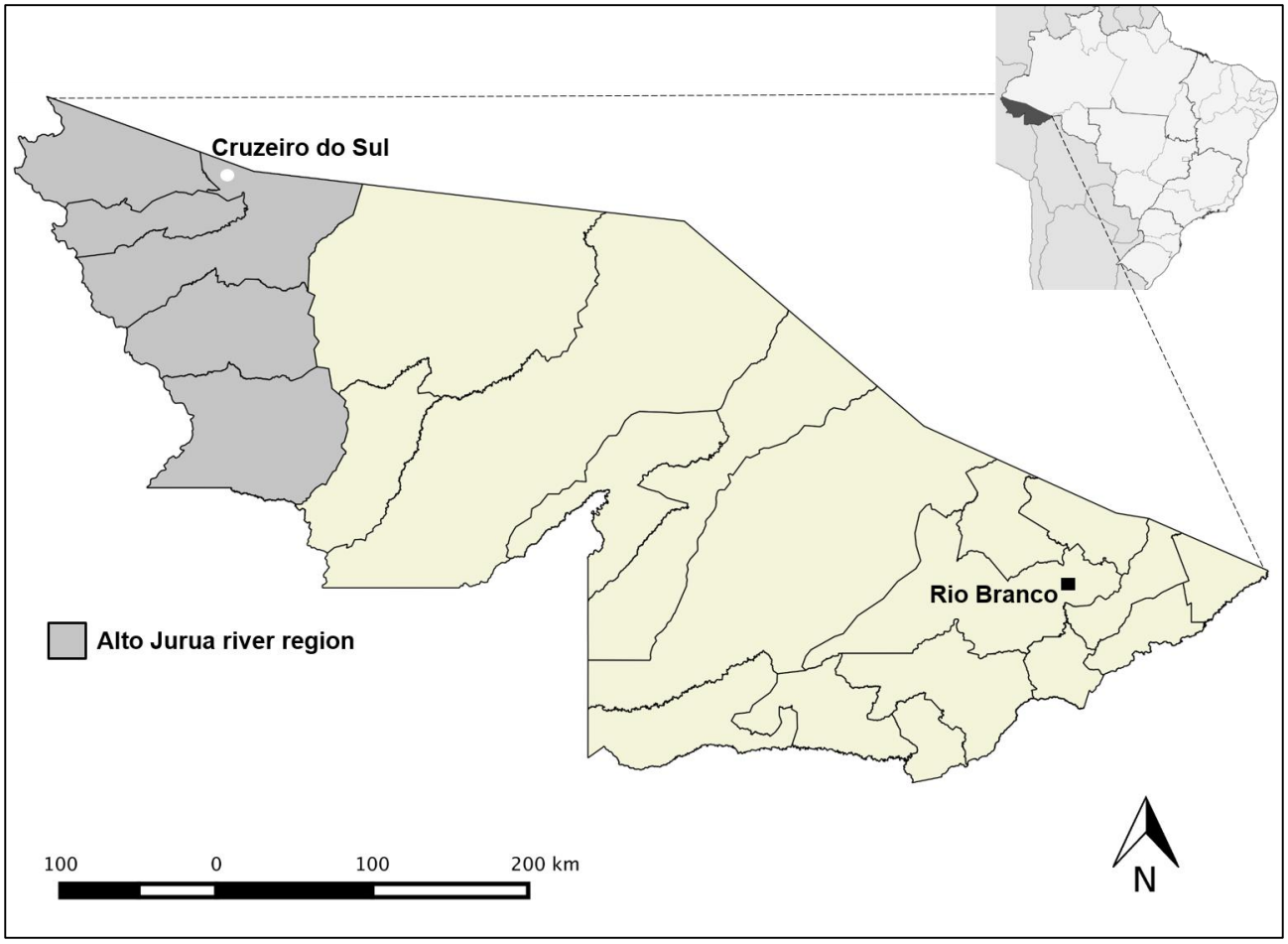
647

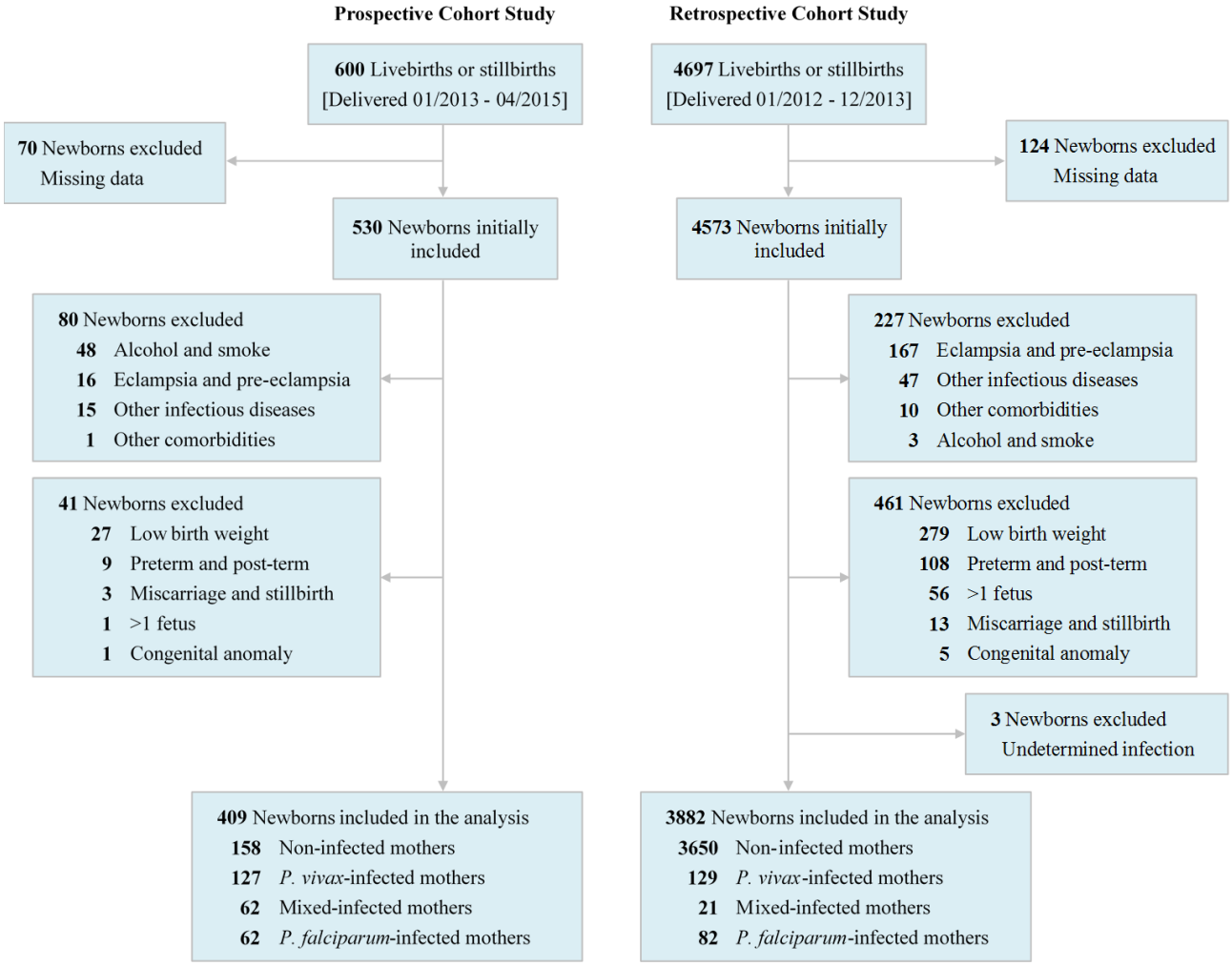
FIGURES

Figure 1. Map showing the location of the field site, Alto do Juruá river region, Northwest of the Acre State, Brazilian Amazon.



The map also indicates Cruzeiro do Sul where the field laboratory is situated, and Rio Branco, the capital of the state of Acre.

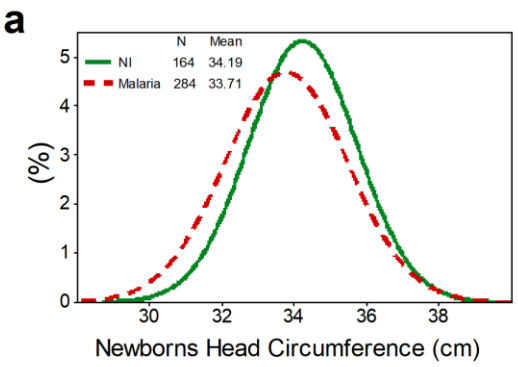
Figure 2. Flow diagram of the two cohort studies detailing exclusion criteria.

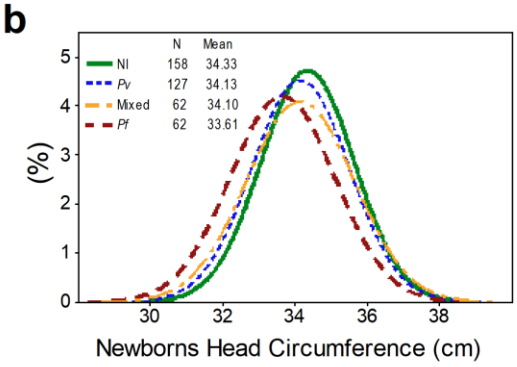


Mixed infection – *P. vivax*- and *P. falciparum*-infection occurring at the same time and/or at different times during pregnancy.

676 **Figure 3. Prospective cohort study shows that malaria infection during pregnancy impacts**

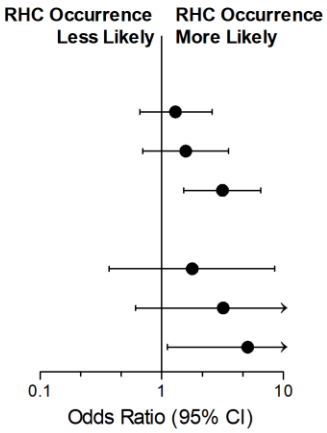
677 **babies head circumference.**





c

Newborns	n/N	Prevalence (%)	Odds Ratio (95% CI)	*P*-Value
Small Head (HC < -1 SD)	70/409	17.1		
P. vivax	20/127	15.8	1.30 (0.66 - 2.59)	0.449
Mixed	11/62	17.7	1.58 (0.70 - 3.55)	0.272
P. falciparum	19/62	30.7	3.15 (1.52 - 6.53)	0.002
Microcephaly (HC < -2 SD)	15/409	3.7		
P. vivax	4/127	3.2	1.78 (0.37 – 8.46)	0.469
Mixed	3/62	4.8	3.21 (0.61 - 16.77)	0.168
P. falciparum	5/62	8.1	5.09 (1.12 – 23.17)	0.035



678

679 **a**, **b** Newborns head circumference frequency distribution in the PCS according to maternal
680 infection status: malaria- and non-infected (NI) mothers (p = 0.005) (**a**), and NI, *Pv*, Mixed and *Pf*-
681 infected mothers after excluding LBW and preterm babies (NI vs *Pf* p = 0.023) (**b**). The differences
682 in the frequency distributions between each group were examined with Mann-Whitney rank sum
683 tests. **c** Forest plot of the Odds Ratio of small head or microcephaly in babies born from women
684 infected during pregnancy compared to babies from non-infected women, according to *Plasmodium*
685 species. Mixed infection – *P. vivax*- and *P. falciparum*-infection occurring at the same time and/or
686 at different times during pregnancy. n/N - number of events by total number of individuals in each
687 group; CI - confidence interval; HC - head circumference; SD - standard deviation; *P*-Values were
688 estimated through multivariate logistic regression methods.

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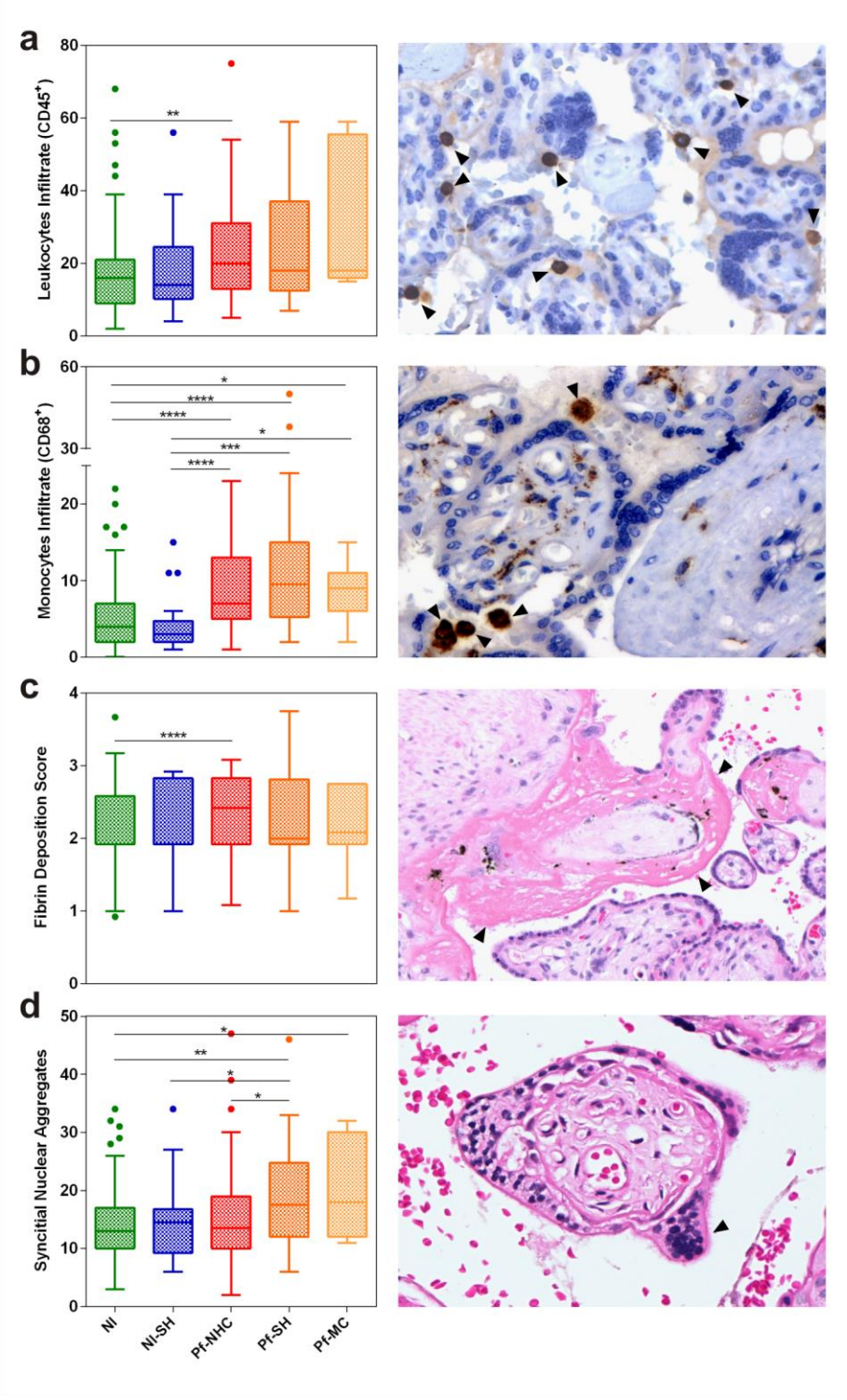
692 **Figure 4. Histopathological parameters evaluation of placentas from non- and *P. falciparum*-**

693 **infected mothers according to newborns head circumference.**



694

695 **a** Leukocytes ($CD45^+$) number. **b** Monocytes ($CD68^+$) number. **c** Fibrin deposition score. **d**

696 Syncytial nuclear aggregates. Images in each panel are only representative. Histopathological

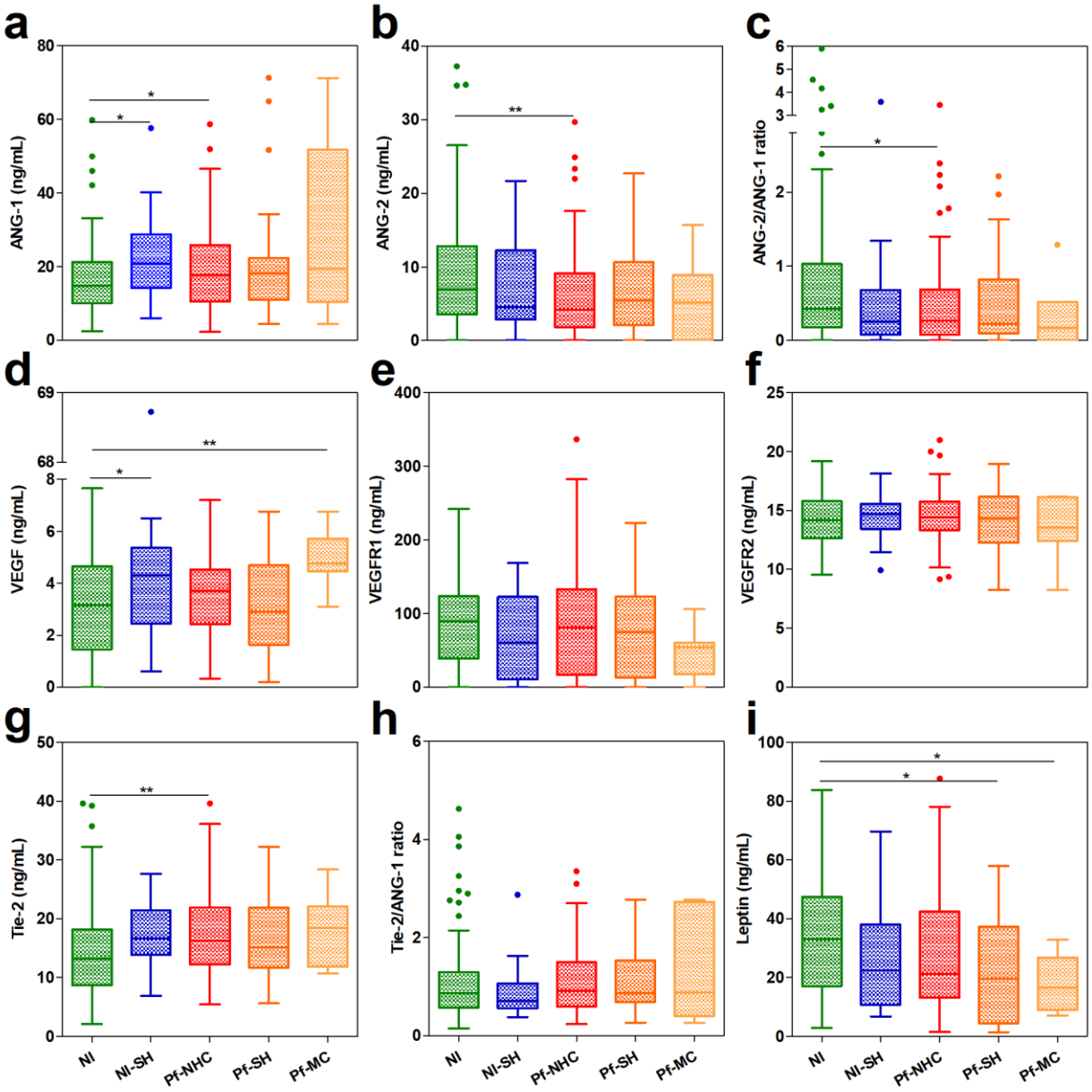
697 parameters were evaluated by microscopy through H&E (fibrin deposition and syncytial nuclear

698 aggregates) and immunohistochemistry (leukocytes and monocytes) staining. NI – non-infected;

699 NI-SH – non-infected small head; Pf-NHC – *P. falciparum*-infected normal head circumference; Pf-

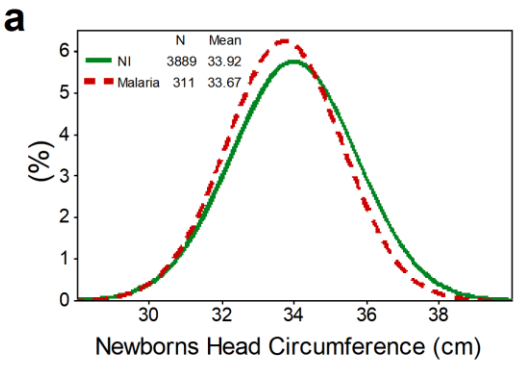
SH - *P. falciparum*-infected small; and, Pf-MC - *P. falci*parum-infected microcephaly. Data are represented as Tukey boxplots, the bottom and the top of the box are the first and third quartiles, the line inside the box is the median, and the whiskers represent the lowest and the highest data within 1.5 IQR of the first and upper quartiles. The differences between each group were examined with Mann-Whitney rank sum tests, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

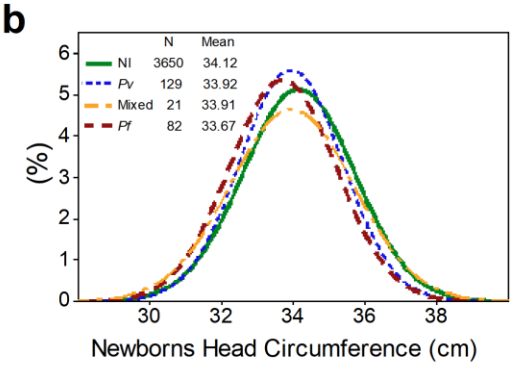
Figure 5. Placental plasma levels of angiogenic factors and leptin from non- and *P. falciparum*-infected mothers according to newborns head circumference.



a Angiopoietin-1 (ANG-1). **b** Angiopoietin-2 (ANG-2). **c** Ratio ANG-2/ANG-1. **d** Vascular endothelial growth factor (VEGF). **e** VEGF receptor-1 (VEGFR-1). **f** VEGF receptor-2 (VEGFR-2). **g** TEK receptor tyrosine kinase (Tie-2). **h** Ratio Tie-2/ANG-2. **i** Leptin. All factors were measured by ELISA. NI – non-infected; NI-SH – non-infected small head; Pf-NHC – *P. falciparum*-infected normal head circumference; Pf-SH - *P. falciparum*-infected small; and, Pf-MC - *P. falci*parum-infected microcephaly. Data are represented as Tukey boxplots, the bottom and the top of the box are the first and third quartiles, the line inside the box is the median, and the whiskers represent the lowest and the highest data within 1.5 IQR of the first and upper quartiles. The differences between each group were examined with Mann-Whitney rank sum tests, * $p \leq 0.05$, ** $p < 0.01$.

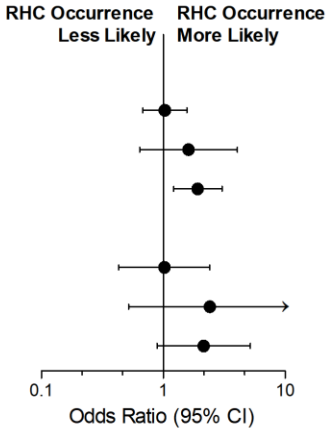
Figure 6. Retrospective cohort study corroborates that malaria infection during pregnancy impacts babies head circumference.





c

Newborns	n/N	Prevalence (%)	Odds Ratio (95% CI)	*P*-Value
Small Head (HC < -1 SD)	934/3882	24.1		
P. vivax	32/129	24.8	1.03 (0.68 – 1.56)	0.88
Mixed	7/21	33.3	1.61 (0.64 – 4.05)	0.31
P. falciparum	30/82	36.6	1.91 (1.21 – 3.04)	0.006
Microcephaly (HC < -2 SD)	161/3882	4.2		
P. vivax	6/129	4.7	1.02 (0.43 - 2.40)	0.96
Mixed	2/21	9.5	2.41 (0.52 – 11.24)	0.26
P. falciparum	6/82	7.3	2.14 (0.89 – 5.19)	0.09



a, **b** Newborns head circumference frequency distribution in the PCS according to maternal infection status: malaria- and non-infected (NI) mothers (p = 0.008) (**a**), and NI, *Pv*, Mixed and *Pf*-infected mothers after excluding LBW and preterm babies (NI vs *Pf* p = 0.015) (**b**). The differences in the frequency distributions between each group were examined with Mann-Whitney rank sum tests. **c** Forest plot of the Odds Ratio of small head or microcephaly in babies born from women infected during pregnancy compared to babies from non-infected women, according to *Plasmodium* species. Mixed infection – *P. vivax*- and *P. falciparum*-infection occurring at the same time and/or at different times during pregnancy. n/N - number of events by total number of individuals in each group; CI - confidence interval; HC - head circumference; SD - standard deviation; p-Values were estimated through multivariate logistic regression methods.

Table 1. Infection characteristics in *P. falciparum*-infected pregnant women.

	***Pf*-NHC (N=94)**	***Pf*-SH (N=30)**	***p*-Value[a]**	***Pf*-MC (N=8)**	***p*-Value[b]**
Infections per pregnancy, median (IQR)	2.0 (1.0-3.0)	2.0 (1.0-2.0)	0.463	1.0 (1.0-2.0)	0.116
Parasitemia of first infection, median (IQR)[c]	1.2 (0.3-4.6)	3.8 (0.5-9.2)	0.053	0.4 (0.2-1.8)	0.220
Gestational age at first infection					
Mean (SD)	20.7 (10.5)	26.0 (8.1)	0.014	27.6 (7.8)	0.064
Median (IQR)	19.0 (12.0-29.3)	25.5 (18.0-32.5)		28.5 (19.8-34.3)	
Placental Malaria, no. (%)[d]					
No	29 (36)	7 (30)	-	1 (14)	-
Active Acute	8 (10)	2 (8)	-	0	-
Active Chronic	5 (6)	2 (8)	-	1 (14)	-
Past	38 (48)	13 (54)	-	5 (72)	-
Hemozoin, no. (%)[d, e]					
No	31 (39)	8 (33)	-	1 (14)	-
Mild	32 (40)	9 (38)	-	4 (57)	-
Moderate	15 (19)	7 (29)	-	2 (29)	-
Severe	2 (2)	0	-	0	-

N, number of individuals; *Pf*-NHC, *Plasmodium falciparum*-normal head circumference; *Pf*-SH, *Plasmodium falciparum*-small head; *Pf*-MC, *Plasmodium falciparum*-microcephaly; IQR, interquartile range; SD, standard deviation; no., number of events.

[a] Differences between *Pf*-NHC and *Pf*-SH groups were evaluated using Mann-Whitney rank sum tests.

[b] Differences between *Pf*-NHC and *Pf*-MC groups were evaluated using Mann-Whitney rank sum tests.

[c] Parasitemia was recorded in 82 *Pf*-NHC, 28 *Pf*-SH, and 7 *Pf*-MC. Values presented in 10^3 DNA copies.

[d] Placental malaria and Hemozoin was recorded in 80 *Pf*-NHC, 24 *Pf*-SH, and 7 *Pf*-MC.

[e] Hemozoin - Mild: focal presence in small amounts; Moderate: small spots or larger deposits in many locations; Severe: large amounts present widely.