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Original Article

Assessment of the impact of pregnancy and malaria infection on the variation of neutrophil levels in women from San, Mali

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Abstract

Background: In patients with severe neutropenia, infections can rapidly become serious and life-threatening. It is essential to understand whether pregnancy induces changes in neutrophil levels thereby posing an increased threat to the health of gravidae.
Methodology: This cross-sectional study was conducted in San Health District (Mali) and involved pregnant women infected or not by malaria parasites and non-pregnant healthy volunteers. Subjects were categorized as having neutropenia, normal neutrophil levels, and neutrophilia regarding their neutrophil levels. A logistic regression analysis was performed to determine factors associated with neutrophil level variation in pregnant women.
Results: Whether or not the pregnant women were infected with malaria, 98 of the 202 cases (48.5%) showed neutrophilia. Surprisingly, 67 of the 71 cases of neutropenia (94.4%) observed in this study concerned healthy people who were not pregnant. The mean percentage of neutrophil levels was significantly ($p < 0.001$) lower (49.9%) in the first trimester compared to the second trimester of pregnancy (62.0%). A logistic regression model showed that compared to early pregnancy, the second (OR = 12.9, 95% CI 2.2-248.1, $p = 0.018$) and the third trimesters (OR = 13.7, 95% CI 2.3-257.5, $p = 0.016$) were strongly associated with the increase of neutrophil levels.
Conclusions: Pregnancy can induce the production of mature neutrophils that are continually released into circulation. Neutrophil levels were lower during the first trimester of the pregnancy compared to the second and third trimesters, but not affected by the presence or absence of malaria infection.

Key words: Neutrophil; pregnancy; malaria; Mali.

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Introduction

Neutrophils are essential components of the innate immune response and a major contributor to inflammation. They are a specific class of white blood cells (WBC), also known as polymorphonuclear leukocytes (PMNs), which are phagocytic cells capable of direct antimicrobial activity and immune regulation [1]. They appear to offer the first line of immune defense and act as regulators of innate and adaptive immunity [2,3]. Consequently, the neutrophil level in a person is an important indicator of whether that individual can start combating infectious diseases. Severe neutropenia can have life-threatening implications [4]. However, there seems to be a difference amongst populations on what effect normal or decreased levels of neutrophils have on immunity. For example, people of African descent appear to have low absolute neutrophil counts compared to Caucasian and white American people but these low levels are described as benign [5–8]. Another phenomenon in which neutrophil levels fluctuate is during pregnancy. Neutrophils increase in peripheral blood in pregnant

compared to healthy women. Their counts gradually and significantly increase from the beginning of the pregnancy to the 3rd trimester [9].

Malaria caused by *Plasmodium falciparum* parasites is the principal cause of morbidity and mortality particularly in pregnant women and children under 5 years old in Mali [10,11]. In malaria-endemic areas, pregnant women are more vulnerable to malaria than non-pregnant women. This is in part due to immunological and hormonal changes that occur during pregnancy [12]. Furthermore, the ability of a subset of *P. falciparum*-infected erythrocytes to sequester in the placenta contributes to severe disease [13]. Although malaria during pregnancy is mostly asymptomatic, it increases the risk of maternal anemia, preterm deliveries, stillbirth, and low birth weight babies [12].

During pregnancy-associated malaria (PAM), *P. falciparum*-infected erythrocytes sequester in the placenta [14], often resulting in monocytic infiltration with increased production of pro as well as anti-inflammatory mediators [15,16]. In pregnant women, the role of neutrophils in malaria pathogenesis remains poorly understood. However, there is scientific evidence suggesting that neutrophil infiltration, activation, and dysfunction may worsen malaria outcomes or predispose to other bacterial infections [17–19]. One study reported that the presence of malaria pigment in circulating neutrophils was associated with decreased birth weight [20]. It has also been described that pregnant women with *P. falciparum* infection had lower circulating neutrophil levels than uninfected women [21] and that neutrophil activation increases in proportion to the severity of malaria [22]. However, the role of neutrophils in PAM remains insufficiently elucidated.

The present study was undertaken to investigate whether the decrease in immunity during pregnancy affects neutrophil levels in women living in an area of intense and seasonal malaria transmission. To answer this research question, women living in the San health district in Mali were recruited and their neutrophil levels were measured and compared according to the presence or absence of pregnancy. In addition, it was studied if pregnant women who are infected with *P. falciparum* malaria have increased neutrophil levels and if there is an association between parasite density and neutrophil levels within these women.

Methodology

Study site

This study, involving pregnant women and healthy non-pregnant female and male volunteers, was conducted in San Health District, Mali. San is located at latitude 13°18′12″ North and longitude: 4°53′44″ West and at 285 meters elevation above sea level. The city is 440 kilometers (273 miles) from Bamako, the capital city of Mali. The study area experiences malaria transmission throughout the year with a peak in October.

Study design

This study was ancillary to the "Efficacy and Safety of Pyronaridine-Artesunate for the treatment of uncomplicated malaria in African pregnant women (PYRAPREG)" clinical trial (PACTR202011812241529, a European & Developing Countries Clinical Trials Partnership (EDCTP) funded project, grant number: RIA2017MC-2025-PYRAPREG, website: https://www.pyrapreg.org/about-project/). The PYRAPREG study is evaluating the efficacy and safety of a newly registered artemisinin-based combination (pyronaridine-artesunate) compared to artemether-lumefantrine or dihydroartemisinin-piperaquine for the treatment of uncomplicated malaria in African pregnant women.

Pregnant women screened for PYRAPREG trial were recruited for the present study regardless of their *P. falciparum* infection status. Infection status was first established by testing with a malaria rapid diagnostic test (RDT) and was subsequently confirmed by two independent microscopy readings. To compare neutrophil levels in the non-pregnant population with those of pregnant women, non-pregnant healthy volunteers (predominantly male 143 out of 160, representing 89.4%) were enrolled from regular blood donors. Like the pregnant women, they were tested for malaria and were all found not infected by microscopy. General demographic data was collected from all participants. Female volunteers were asked about their obstetrical history.

Sample size

The sample size was calculated to compare the frequency of neutropenia in pregnant women infected with malaria parasites to pregnant women not infected with malaria parasites in one arm and to healthy non-pregnant subjects in the other arm. We used the following assumptions: a power of 80% and a confidence interval of 95%. A previous study by our research team [23] showed that the frequency of neutropenia in the children infected by malaria parasite was 25%. We assumed that a 15% increase in this rate in malaria-infected pregnant women would be a

clinically significant increase. Based on these assumptions, a minimum sample size of 152 study participants would be required per group. Assuming 5% uninterpretable data, 160 study subjects were needed per group. Thus, a total of 480 study cases are needed for the present study.

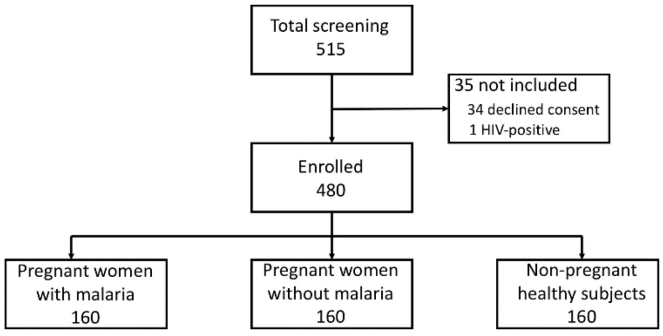
Laboratory analysis

The CareStart™ Malaria *Pf* histidine-rich protein 2 (HRP2) antigen-based RDT was used to screen for malaria infection and to determine persistent antigen after *P. falciparum* clearance. For malaria diagnosis, thick blood smears were stained in 10% Giemsa solution for 15 minutes. Dried slides were examined under an Olympus CX33 microscope (Olympus Corporation, Tokyo, Japan) and parasitemia was estimated by reporting the number of parasites per μL of blood based on the count of 8000 WBC.

From all participants, a venous blood sample was collected and analyzed using CELL-DYN Emerald 22 analyzer (https://www.corelaboratory.abbott/int/fr/offerings/brands/cell-dyn/cell-dyn-emerald22) to determine the full hemogram including hemoglobin level. Blood was taken from malaria-positive patients prior to treatment.

The number of white blood cells was measured per microliters (μL) of blood. Neutrophil levels in WBC count were given as a percentage. Depending on neutrophil levels, subjects were defined as neutropenia, normal level of neutrophils, or neutrophilia. In addition, the study participants were defined as having leukopenia, normal WBC count, or having hyperleukocytosis. Definition of WBC and neutrophil levels normal ranges were based on the following cut-off points [24] which are quite close to the unpublished standards in Mali: Leukopenia (WBC < 4,500); Normal WBC count (4,500 ≤ WBC count ≤ 11,000); Hyperleukocytosis (WBC count > 11,000); Neutropenia (neutrophil levels < 40%); Normal neutrophil levels (40% ≤ neutrophil levels ≤ 60%) and Neutrophilia (neutrophil levels > 60%).

Figure 1. Trial profile. Total number of volunteers screened, the number included and the reasons for failure to include. Distribution of the study participants in the different arms.



Statistical analysis

Study participants were divided into three groups based on pregnancy status and malaria infection, i.e., pregnant women with confirmed *falciparum* malaria infection, pregnant women without malaria, and non-pregnant healthy subjects (Figure 1). To assess whether PAM modifies neutrophil levels, the following analysis strategy was carried out: descriptive analysis of the 3 study arms; descriptive analysis of WBC and neutrophil levels; status of neutrophil levels during pregnancy and factors associated with increased neutrophil levels in pregnant women.

Analyses were focused on WBC as a whole (leucocytes) and neutrophils specifically, measured as count per microliter (μL) of blood and percent of WBC counts, respectively.

Cross-tables were used to assess the frequencies of the categories of WBC count and neutrophil levels by study arms. To compare the proportions of WBC and neutrophil levels over the different groups a chi-square or Fisher test was used where appropriate. Hemoglobin level was also compared between study groups. A Logistic regression analysis was performed to identify factors associated with neutrophilia in pregnant women. Beeswarm plots [25] were used to visualize the variation in neutrophil levels between study arms in general and the variation in neutrophil levels by pregnancy age in pregnant women. The average neutrophil levels were compared two by two between study arms with student's t-test or Wilcoxon test where applicable. ANOVA was used when we had to compare more than 2 groups. A p value < 0.05 was deemed statistically significant. Statistical analyses were performed using R software (version 3.6.3) using libraries "descr" for crosstables, "aod" regression analysis and "beeswarm" for plots.

Results

Study profile and general characteristics

From an overall 515 individuals screened, 480 were enrolled in this study. Of those excluded from the study, 35 refused the venous sampling essential for this analysis and one woman tested positive for human immunodeficiency virus during her prenatal check-up (Figure 1).

Participants comprised 160 pregnant women infected by *P. falciparum*, 160 pregnant women non-infected, and 160 controls (143 healthy males and 17

healthy females). In addition, there was a non-pregnant healthy population (n = 160) which predominantly consisted of male participants (143 out of 160 (89.4%)) (Table 1). The average age (33.0 ± 8.4 years) of the non-pregnant healthy group was significantly higher compared to the group of pregnant women with malaria (22.6 ± 5.3 y, $p < 0.001$) and the group of pregnant women without malaria (24.6 ± 5.8 y, $p < 0.001$). In terms of age distribution, 103 out of 160 (64.4%) of the non-pregnant population were 30 years or older. However, only 18 out of 160 (11.2%) and 37 out of 160 (23.1%) were 30 years or older in the group of pregnant women with malaria and pregnant women without malaria, respectively. In the group of healthy non-pregnant subjects, a sub-analysis showed that the mean age of the men was 33.2 years and that of the women was 30.9. Although the women were relatively younger in this group, the difference was not statistically significant ($p = 0.287$). For gravidity, nearly half of all the women who participated in this analysis had at least 3 pregnancies (156 out of 337; 46.3%).

For those women who were confirmed to have a malaria infection, the average *P. falciparum* parasite count was 10925 ± 22614 per µL of blood.

Descriptive analysis of WBC and neutrophil levels

WBC mean count (4416; SD = 1314) was lower in healthy non-pregnant subjects compared to pregnant women with malaria infection (5493, SD = 1529) and pregnant women without malaria infection (7674; SD =10516) ($p < 0.001$) (Table 1).

Table 2 shows the variation in WBC count in general and neutrophil levels in particular in the three study groups. In the group of non-pregnant individuals, there was a significantly higher proportion of people with leukopenia (65 out of 95 in total, 68.4%) compared to pregnant women without malaria (8 out of 95 in total, 8.4%) and pregnant women with malaria (22 out of 95 in total, 23.2%) ($p < 0.001$).

Table 2 shows that neutrophilia was most frequently observed in pregnant women, whether or not they were infected with malaria parasites. A total of 202 of the participants in this study had neutrophilia of which we have an equal number (98 of 202, 48.5%) of pregnant women in the malaria-infected and non-malaria-infected groups. In healthy non-pregnant individuals, 67 cases of neutropenia were observed out of the 71 cases observed in total (94.4%). A sub-analysis of only the predominantly male non-pregnant individuals shows that both WBC and neutrophil levels are higher in healthy non-pregnant women compared to healthy men ($p < 0.05$) (Figure 2).

The mean percentage of neutrophil levels in the WBC count was significantly lower in healthy non-pregnant subjects (41.8%) compared to pregnant women without malaria (61.4%) and pregnant women

Table 1. General characteristics of the study population.

Characteristics	Study Group			Overall (n = 480)
	Non-pregnant healthy group (n = 160)	Pregnant women with malaria (n = 160)	Pregnant women without malaria (n = 160)	
Gender, n (%)				
Female	17 (10.6)	160 (100)	160 (100)	337 (70.2)
Male	143 (89.4)	-	-	143 (29.8)
		p < 0.001		
Age, y, mean (SD) [Min, Max]	33.0 (8.4) [15–74]	22.6 (5.3) [15–37]	24.6 (5.8) [15–45]	26.7 (8.0) [15–74]
		p < 0.001		
Age* group, y, n (%);				
< 20	4 (2.5)	60 (37.5)	34 (21.2)	98 (20.4)
20 to 29	53 (33.1)	82 (51.2)	89 (55.6)	224 (46.7)
> 29	103 (64.4)	18 (11.2)	37 (23.1)	158 (32.9)
		p < 0.001		
Gravidity, n (%)				
Nulligest	4 (23.5)	-	-	4 (1.2)
Primigravida	4 (23.5)	61 (38.1)	35 (21.9)	100 (29.7)
2nd pregnancy	4 (23.5)	38 (23.8)	35 (21.9)	77 (22.8)
Multigravida	5 (29.4)	61 (38.1)	90 (56.2)	156 (46.3)
		p < 0.001		
RDT result, n (%)				
Positive	0 (0.0)	160 (100)	11 (6.9)	171 (35.6)
Negative	160 (100)	0 (0.0)	149 (93.1)	309 (64.4)
		p < 0.001		
Asexual parasite, per µL, mean (SD) [Min, Max]	-	10925 (22613.6) [40–131520]	-	-
WBC count, per µL, mean (SD) [Min, Max]	4416.3 (1313.8) [1800–9000]	5493.2 (1528.6) [1310–11300]	7673.8 (10515.6) [2800–83000]	5861 (6316.2) [1310–83000]
		p < 0.001		

Y: year; µL: microliter; SD: standard deviation; WBC: white blood cell; Min: Minimum; Max: Maximum.

Table 2. WBC count and neutrophil levels distribution according to study arms.

Characteristics	Study Group			Overall (n= 480)
	Healthy non-pregnant (n = 160)	Pregnant with malaria (n = 160)	Pregnant without malaria (n = 160)	
WBC count status, n (%)				
Leukopenia	65 (68.4)	22 (23.2)	8 (8.4)	95 (19.8)
Normal WBC count	95 (25.0)	137 (36.1)	148 (38.9)	380 (79.2)
Leucocytosis	0 (0.0)	1 (20.0)	4 (80.0)	5 (1.0)
Neutrophil status, n (%)				
Neutropenia	67 (94.4)	1 (1.4)	3 (4.2)	71 (14.8)
Normal level	87 (42.0)	61 (29.5)	59 (28.5)	207 (43.1)
Neutrophilia	6 (3.0)	98 (48.5)	98 (48.5)	202 (42.1)

WBC: white blood cell.

with malaria infection (62.9%) ($p < 0.001$). However, the difference was not statistically significant between malaria-infected pregnant women and pregnant women without malaria ($p = 0.178$) (Figure 3).

Neutrophil levels during pregnancy

During pregnancy, the level of neutrophils change according to trimester. Figure 4 shows that the means

Figure 2. White Blood Cells and neutrophils in non-pregnant study subjects. In non-pregnant healthy subjects, **A.** the mean number WBC count as well as **B.** the mean percentage of neutrophil level are higher in female compared to men.

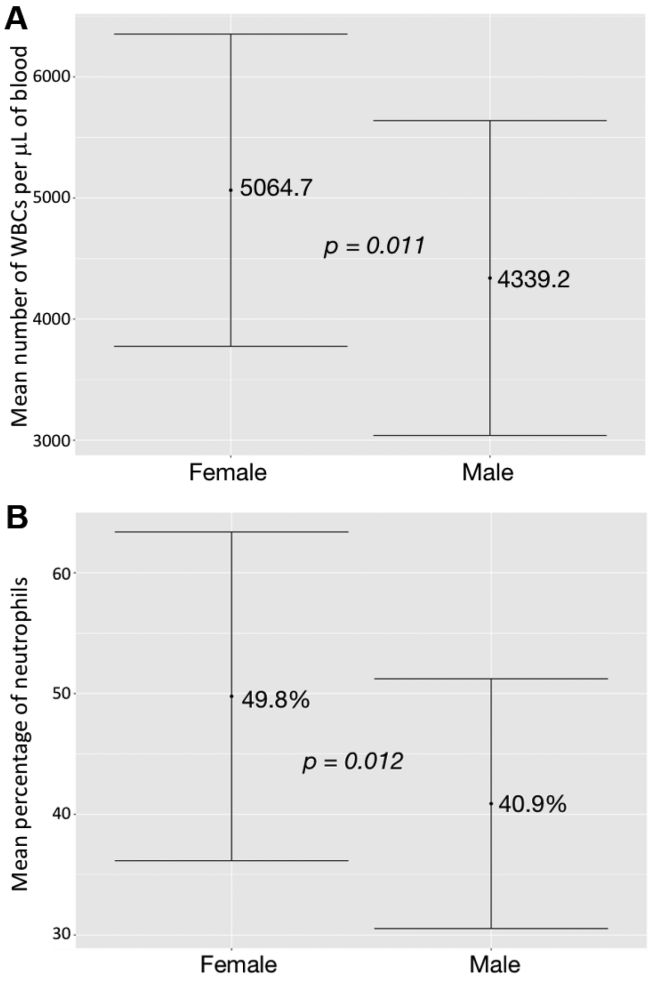


Figure 3. Neutrophil levels according to pregnancy and malaria infection. Each point representing individuals' neutrophil rate, the graph allows for quick quality control. The middle line of the boxplot represents the median, and the upper and lower bounds of the box are the 75th and 25th percentiles respectively. The graph shows that there are as many samples per group and that the mean neutrophil levels is lower in the non-pregnant group.

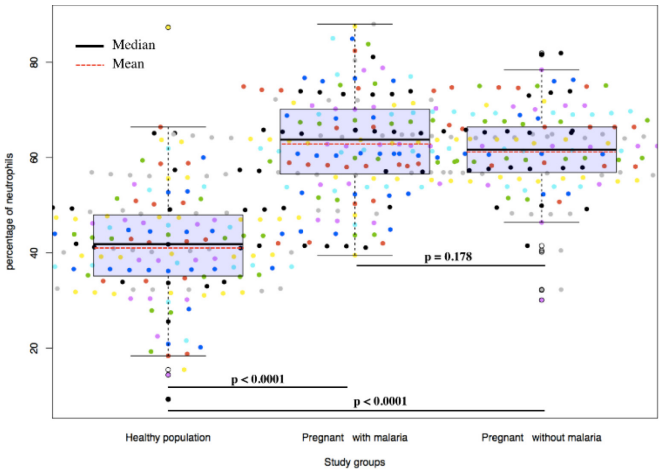
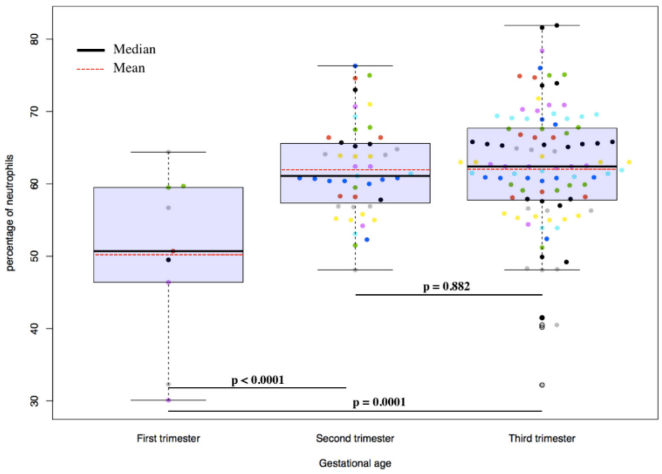


Figure 4. Neutrophils level according to gestational age. Each point representing the individual neutrophil rate shows that there are few women in the first trimester of pregnancy. Also, the median, and interquartile range (75th and 25th percentiles) are lower in those women in the first trimester of pregnancy.



neutrophil levels in the first trimester (49.9%) increase to 61.4% in the second trimester of pregnancy. This is a statistically significant increase of 11.5% ($p < 0.001$). Between the second (61.4%) and third trimester of pregnancy (62.2%), the mean percentage of neutrophil levels in the WBC count increased slightly (0.8%), but this was not significant ($p = 0.882$). A sub-analysis of only the malaria-infected women data shows that their mean neutrophil levels were 62.2% and 64.1% during second and third trimester of pregnancy ($p = 0.3$).

Factors associated with increased neutrophil levels in pregnant women

The logistic regression model identified factors associated with increased neutrophil levels (neutrophilia) in pregnant women (Table 3). Compared to the first trimester of pregnancy, the second (OR = 12.98, 95% CI 2.2-248.1, $p = 0.0187$) and the third trimester (OR = 13.56, 95% CI 2.3-257.5, $p = 0.0161$) are strongly associated with neutrophilia. The model shows that with each increase of one parasite in parasitemia the chance of developing neutrophilia multiplies by 1.00003 (OR = 1.00003, 95% CI 1.0-1.0, $p < 0.0203$). Women with positive malaria RDT did not show an association with neutrophilia (OR = 1.46, 95% CI 0.4-7.0, $p = 0.5947$) compared to women with negative RDT.

Discussion

This study aimed to investigate the association between neutrophil levels during different trimesters of pregnancy in women with and without malaria infection. The results showed that pregnant women have higher WBC counts compared to non-pregnant non-infected participants. In addition, pregnant women without malaria have higher WBC counts compared to pregnant women with malaria. Also, an increase in neutrophil levels was observed in pregnant women compared to non-pregnant non-infected individuals. This study confirms previous data showing that the second and the third trimesters of pregnancy are strongly associated with an increase in neutrophil levels [9].

Pregnant women with malaria were found to have lower leukocyte counts compared to pregnant women without malaria. This is in line with a previous study, by McKenzie *et al.,* which has shown that often, in cases of malaria infection, the total number of leukocytes appears low or normal, rather than elevated [26]. According to the same study, this may reflect the localization of leukocytes out of the circulation into the spleen and other marginal pools, instead of the actual depletion of leukocytes [26].

During pregnancy, regardless of malaria status, there is a progressive increase in the normal WBC count, as well as a slight shift towards an increased percentage of neutrophils [9]. Our data do not show a statistically significant change in neutrophil levels depending on whether the pregnant woman is infected with *P. falciparum* or not. Besides malaria, other infections in pregnant women have been shown to increase the neutrophil levels [27]. Our previous study [23] has shown that in children, malaria infection causes an increase in the neutrophil levels which decreases drastically 3 days after anti-malaria treatment. Although children are also vulnerable to malaria as are pregnant women, the age difference could play a role in the activation of immunity.

Healthy non-pregnant and non-malaria-infected subjects (predominantly consisting of males) were found to have both leukopenia and neutropenia in our present study. This is in line with observations that descendants of black Africans tend to have a slight depletion of WBC counts and absolute neutrophil levels compared to non-black Africans [28]. When these non-pregnant participants are compared to pregnant women our data shows an increased WBC and neutrophil levels in pregnant women. This study provides evidence that neutropenia among individuals of African descent [6] does not apply to pregnant women, especially from the 2nd trimester and onwards.

Our data showed that even in the absence of pregnancy and malaria infection, the proportion of the subjects with neutropenia is higher in men compared to women. The relatively more frequent fungal infections in women may contribute to a greater release of

Table 3. Logistic regression model showing factors associated with neutrophilia in pregnant women.

Characteristics	Odd ratio	Confidence interval		*p* value
		Lower limit	Upper limit	
Absence of malaria infection	2.05	0.56	9.73	0.3073
Gestation at second trimester	12.98	2.20	248.10	0.0187*
Gestation at third trimester	13.56	2.34	257.48	0.0161*
Primigravida	1.00	0.58	1.76	0.9877
Second pregnancy	0.71	0.39	1.29	0.2639
Malaria parasite density	1.00	1.00	1.00	0.0203*
Presence of malaria infection (RDT positive)	1.46	0.39	6.96	0.5947

*Statistically significant.

neutrophils into the peripheral blood. Other researchers have shown that women under 50 years old have a higher number and percentage of neutrophils than men [29].

A major limitation of this study is the composition of the control group to assess the neutrophil levels in non-pregnant individuals. This control group was predominantly male. Also, the fact that they are on average ten years older than the two groups of women in the study, i.e., the group pregnant and infected with *Plasmodium* or pregnant and non-infected, could have influenced the observed neutrophil levels. The controls were randomly selected from a pool of blood donors, which is dominated by men because women do not tend to donate blood in our study setting. Despite the under-representation of women in the non-pregnant group, our results comparing neutrophil levels by gender, in line with previous observations [29], demonstrated that neutrophil levels are generally higher in women.

Another limitation of the present study is the under-representation of certain subgroups such as women in the first trimester of pregnancy. A longitudinal study from early pregnancy onwards might ensure a more balanced distribution of women with and without malaria during the different trimesters and the natural neutrophil dynamics over the total course of pregnancy when a malaria infection has occurred.

We have not been able to analyze the neutrophil levels according to the severity of malaria because all our cases were either asymptomatic or uncomplicated, due to the low parasite density in the malaria-infected pregnant women group.

The main strength of this study is the participation of healthy non-pregnant subjects. Although they were mainly male, it provided basic information on the blood count in general and the WBC count in particular healthy people in Mali. This allowed a comparison with actual local baseline data to the WBC formula of healthy pregnant women on the one hand and to the WBC formula of pregnant women infected by malaria parasite on the other hand.

Although neutrophilia in non-pregnant individuals is usually associated with an infection this study has shown that in pregnant women the increase of neutrophils is natural and does not give any indication of an infection with *Plasmodium*. This study underlines the importance of considering neutrophil levels within the local context and the natural dynamics of a person (in this case pregnancy) and also considers other parameters besides blood counts when an infection is expected in pregnancy.

Conclusions

Pregnancy can induce the production of neutrophils that are continually released into the circulation. Neutrophil levels were lower during the first trimester of the pregnancy compared to the second and third trimester, but in contrast to what has been observed in children, there was no significant effect observed of malaria infection on neutrophil count in pregnant women.

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We are grateful to Dr N'Fa Adama Diallo, the Head of the Health District of San and his staff for the support in volunteers pre-screening.

Ethics approval and consent to participate

The study protocol was approved by the ethics committee of the University of Sciences Techniques and Technologies of Bamako (N° 2021/163/CE/USTTB, June 28th, 2021). All participants provided signed informed consent before they participated in the study. For study participants under the legal age of 18 years, after the assent of the volunteers, informed consents were obtained from parents or legal guardians.

Authors' contributions

MD, KK, CA, AD, JKT, MV, HMM, PFM and HDFHS were responsible for the conception and design of this study. BK, MD, HD, MK, MDS, BT, BB and MBT contributed to the data collection. MD did statistical analysis and wrote the first draft of the paper. All authors contributed to critical review and approved the final manuscript.

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