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**Sub-microscopic *Plasmodium falciparum* infections in matched peripheral, placental and umbilical cord blood samples from asymptomatic Congolese women at delivery**

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Graphical abstract



## Heading Highlights

- High prevalence of sub-microscopic infection at delivery.
- High genetic diversity of *Plasmodium falciparum* strains in Congo that varies according to peripheral, placental and umbilical cord blood.
- Reduction of multiplicity of infections.

## Abstract

In malaria-endemic areas, most pregnant women are susceptible to asymptomatic *Plasmodium falciparum* infections. We present here the results of a cross-sectional study conducted in Madibou, a southern district of Brazzaville in the Republic of Congo, between March 2014 and April 2015. The main aim was to characterize *P. falciparum* infections. Blood samples corresponding to peripheral, placental and cord from 370 asymptomatic malaria women at delivery were diagnosed for plasmodium infection by thick blood smears (microscopic infection). Sub-microscopic infection was detected by PCR, using the *MSP-2* gene as marker. Microscopic infections were detected in peripheral, placental and cord blood samples with a prevalence of respectively 7.3% (27/370), 2.7% (10/370) and 0%. The negative samples were submitted to sub-microscopic detection, with respective prevalence of 25.4% (87/343), 16.7% (60/360) and 9.4% (35/370) ( $P < 0.001$ ). We further

investigated the genetic diversity of the parasite by characterizing MSP2 allelic families 3D7 (24 distinct alleles) and FC27 (20 distinct alleles). The total number of alleles for these two families were 31, 25 and 19 in peripheral, placental and cord samples respectively. The 3D7 MSP-2 was the predominant allelic family. The multiplicity of infections (MOI) in peripheral (mean  $1.4\pm 0.01$ ; range 1-4), placental (mean  $1.2\pm 0.01$ ; range 1-3) and cord samples ( $1.4\pm 0.01$ ; range 1-3) were similar ( $P = 0.9$ ) and are unaffected by age, gravidity or sulfadoxine-pyrimethamine. These results shown a high prevalence of sub-microscopic infection and a high genetic diversity of *Plasmodium falciparum* strains in Congo. Age, gravidity and doses of preventive treatment based on sulfadoxine-pyrimethamine do not interfere with the multiplicity of infections.

**Keywords:** asymptomatic pregnant women, *Plasmodium falciparum*, genetic diversity, multiplicity of infections, Congo.

## 1. Introduction

Malaria infection during pregnancy is a serious public health issue in sub-Saharan Africa with approximately 30 million pregnant women being at risk of pregnancy-associated-malaria (Dellicour et al., 2010). The symptoms and complications of malaria infection during pregnancy may vary according to the level of malaria transmission in different geographical areas and the level of acquired immunity (Okell et al., 2009). In high-transmission areas, most infections during pregnancy are asymptomatic but still result in poor pregnancy outcome (Steketee et al., 1996; Mockenhaupt et al., 2000; Nosten et al., 2004). A high proportion of individuals living in malaria-endemic areas harbour *P. falciparum* parasites undetectable by microscopy — therefore called sub-microscopic infection — which can nevertheless be identified using molecular tools like PCR (Snounou et al., 1993). Sub-microscopic infection is common during pregnancy and at delivery (Cottrell et al., 2015). To reduce the burden of malaria infections in pregnant women, the World Health Organization (WHO) has recommended a package of interventions, including the use of long-lasting insecticidal nets (LLINs) and administration of sulfadoxine-

pyrimethamine (SP) as intermittent preventive treatment of malaria during pregnancy (IPTp) (WHO, 2005). In the Republic of Congo, a malaria-endemic country in Central Africa with perennial transmission (Trape and Zoulani, 1987), the administration of IPTp-SP (2 or 3 doses) during pregnancy has been in place since 2006. The study conducted in asymptomatic pregnant women in Brazzaville revealed a reduction of the prevalence of *P. falciparum* infection (Ntoumi et al., 2016). However, high resistance to SP has been found in parasites collected from Congolese pregnant women (Koukouikila-Koussounda et al., 2015). The development of an effective vaccine is the one of the major challenges of the fight against malaria. However, this approach is limited by the polymorphic nature of Plasmodium (Mayengue et al., 2004; Francine et al., 2016; Guitard et al., 2010; Cohee et al., 2016). The susceptibility of pregnant women to malaria is explained by the presence of parasite strains that specifically bind to the chondroitin sulphate A (CSA) present in the placental tissue (Fried and Duffy, 1996; Duffy and Fried, 1999). Investigating of genotype and the circulating parasites in pregnant women is interesting for the malaria vaccine (Genton et al., 2002; Takala et al., 2007; Takala and Plowe, 2009). Several previous studies on the antigenic diversity of *P. falciparum* matched in peripheral, placental and umbilical cord blood have shown varied results in different country. Some studies have shown a parasitic overlap found for each of these compartments while others have revealed a subpopulation of peripheral parasites sequestered in the placenta (Kassberger et al., 2002; Mayengue et al., 2004; Fadlelseed et al., 2017; Guitard et al., 2010; Cohee et al., 2016). Recent published work conducted in the same area reported 29 *MSP-2* alleles in isolates from peripheral blood of pregnant women with asymptomatic *P. falciparum* infection at antenatal visits, suggesting a wide diversity of the *P. falciparum* population (Francine et al., 2016). Therefore, the present cross-sectional study was conducted to characterize *P. falciparum* infections matched in peripheral, placental and cord blood among Congolese women at delivery receiving 1, 2 or more doses of sulfadoxine-pyrimethamine.

## 2. Materials and methods

### 2.1. Study site

The study was carried out at Madibou Integrated Health Center, located in the semi-urban area of Makélékélé district, Southern part of Brazzaville, the capital of the Republic of Congo. This area with about 6.000 inhabitants is situated along the Congo River, where malaria transmission is intense and continuous throughout the year. The entomological inoculation rate is approximately 23 infective bites/person/year (Trape and Zoulani, 1987). *P. falciparum* is the predominant malaria species and *Anopheles gambiae* s.s the main mosquito vector.

### 2.2. Study population

Between March 2014 and April 2015, a total of 370 pregnant women were recruited at the maternity ward. The inclusion criteria were as follows: signed informed consent obtained from the participant at admission before delivery, no episodes of clinical malaria during the last two weeks, no notion of fever at least 48 hours before enrolment and an axillary temperature  $\leq 37.5$  ° C during the examination. Socio-demographic data were recorded using a structural questionnaire and from medical records.

### 2.3. Ethics

The study was approved by the Institutional Ethics Committee of the Fondation Congolaise pour la Recherche Médicale (ethical clearance No 001/CEI/FCRM/2012). Written informed consent was obtained from all pregnant women before enrolment.

### 2.4. Blood collection and treatment of samples

A total volume of 5 ml of peripheral blood was collected before delivery, 5 ml placental intervillous and umbilical cord blood were collected within 30 min after delivery into EDTA tubes for the detection of and quantification of malaria parasites. Thick blood smears were

prepared for each blood compartment, stained with 10% Giemsa solution for 15 min, and examined by an experienced microscopist. Trophozoites were counted concomitantly with 200 leukocytes, and expressed in number of asexual parasites per  $\mu\text{L}$  of blood, assuming the leukocyte count of 8.000/ $\mu\text{L}$  of blood. The rest of the blood was spun to separate cells from plasma and stored separately at  $-20^{\circ}\text{C}$  for future use.

## 2.5. DNA extraction and characterization of *P. falciparum* isolates

Genomic DNA was extracted separately from peripheral, placental and cord blood using the QIAmp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer instructions. Parasite isolates were genotyped for *P. falciparum* using the nested PCR technique, targeting the highly polymorphic locus *MSP-2* central region as previously described (Francine et al., 2016). The initial amplifications were followed by individual nested PCR reactions, using specific primers for FC27 and 3D7 allelic families of the *MSP-2* gene. Allele-specific positive controls (3D7 and Dd2) and DNA-free water as a negative control were included in each set of reactions. Each PCR product was stained with Sybr Green, loaded on a 2% agarose gel, and visualized under ultraviolet trans-illumination. Various alleles were determined by their amplicon sizes and the corresponding allele-specific primers used. The sizes of PCR product were estimated using a 100 bp DNA ladder marker (Invitrogen, Karlsruhe, Germany). It was assumed that one band represented one amplified PCR fragment derived from a single copy of *P. falciparum* *MSP-2* gene. Alleles in each family were considered as one if fragment sizes were within a 20 bp interval. The number of different *MSP-2* fragments identified per isolate was considered as the minimum number of parasite genotypes present in this isolate. The amplified PCR products from the different blood compartments for the one woman were run in the same gel to ensure a better comparison.

## 2.6. Case definitions

Asymptomatic *P. falciparum* infection was defined as the presence of malaria parasites in blood in the absence of any clinical symptom. Peripheral, placental and cord blood isolates were grouped into a) *P. falciparum* microscopy positive infection; b) *P. falciparum* infection

that were negative by microscopy but detected by PCR (sub-microscopic infection); c) negative individuals who were negative by both microscopy and PCR. The genetic diversity of *MSP-2* alleles was determined as the total number of different *MSP-2* fragments amplified. The multiplicity of infection (MOI) was defined as the mean number of *P. falciparum* genotypes per infected woman. Gravity was grouped as paucigravidae (First and second full-term pregnancies), and multigravidae (3rd full term pregnancies).

## *2.7. Statistical analysis*

All statistical analyses were performed using R (version 3.5.1) under the RStudio environment (1.1.456). To evaluate the impact of different factors (age, gravity, SP), we applied non-parametric tests, since the multiplicity of infections takes discrete values, and does thus not comply with the normality assumption required for parametric tests. For two-group comparisons (age, gravity) we applied Wilcoxon test. For multi-group comparisons (number of SP doses) we ran a Kruskal-Wallis test. For multi-group comparisons between compartments (prevalence of infection; allelic diversity), we applied the Kruskal-Wallis test. The statistical significance threshold was set to  $P < 0.05$ .

## **3. Results**

### *3.1. Characteristics of the study population*

From March 2014 to April 2015, a total of 370 women were enrolled in the study. The characteristics of the women are shown in Table 1.



**Table 1. Characteristics of the Congolese women at delivery.** The age and gestational age are given as mean plus or minus standard deviation. N = number of women in each group.

Parameters	Values (N = 370)
Mean age $\pm$ SD (years)	25.9 $\pm$ 6.45.
Mean gestational age $\pm$ SD (weeks)	38.1 $\pm$ 1.9
Gravidity, n (%)	
Paucigravidae	152 (41%)
Multigravidae	218 (59%)
Number of IPTp-SP doses	
No	60 (16.2%)
1 dose	106 (28.6)
2 doses	95 (25.7%)
3 doses	112 (30.3%)

### 3.2. Prevalence of *P. falciparum* asymptomatic infection

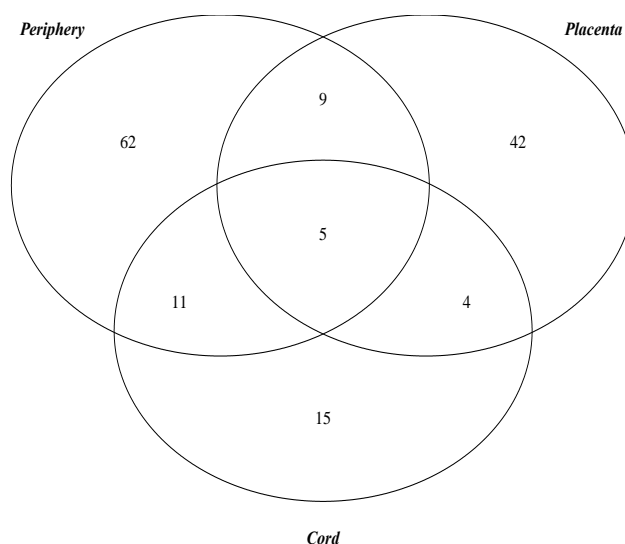
Overall, matched peripheral, placental and cord blood samples from all study participants were analysed. The prevalence of *P. falciparum* microscopic infection in peripheral blood was 7.3% and that of placental blood was 2.7% (Table 2). Not a single sample was found microscopic positive in cord blood (Table 2). The prevalence of *P. falciparum* sub-microscopic infections was 25.4% in peripheral blood, 16.7% in placental blood and 9.4% in cord blood (Table 2). All the microscopy-positive peripheral and placental blood samples were positive by PCR. The overall (microscopy-positive and sub-microscopic infections put together) prevalence of *P. falciparum* sub-microscopic infection was 30.8% in peripheral blood and 19.4% in placental blood (Table 2).

**Table 2. Prevalence of MSP-2 *P. falciparum* infections in Congolese women at delivery.**

N: number of women recruited in this study. For each woman, we collected three blood samples corresponding for peripheral, placenta and cord.

N = 370	Peripheral, n (%)	Placenta, n (%)	Cord, n (%)	P
<b>Infections</b>				
Microscopy	27/370 (7.9)	10/370 (2.7)	0/370 (0.0)	0.005
Sub-microscopy	87/343 (25.4)	60/360 (16.7)	35/370 (9.5)	< 0.001
Total PCR	114/370 (30.8)	70/370 (19.4)	35/370 (9.5)	< 0.001

Out of 87 (25.4%) of women with peripheral sub-microscopic infections, 9 (4.6%) had placental infections and 11 (12.6%) had infections in cord. Out of 60 (16.7%) of women with placental sub-microscopic infections, 4 (6.7%) samples were matched in cord. Furthermore, 62 (71.3%), 42 (70%) and 15 (42.8%) positive samples were detected exclusively in peripheral, placental and cord respectively. Overall, 5 cases of *P. falciparum* sub-microscopic infections matched in peripheral, placental and cord samples (Fig. 1).



**Fig. 1:** Sub-microscopic *P. falciparum* infections in Congolese women at delivery matched in peripheral, placental and cord blood.

### 3.3. Distribution of *P. falciparum* MSP-2 alleles

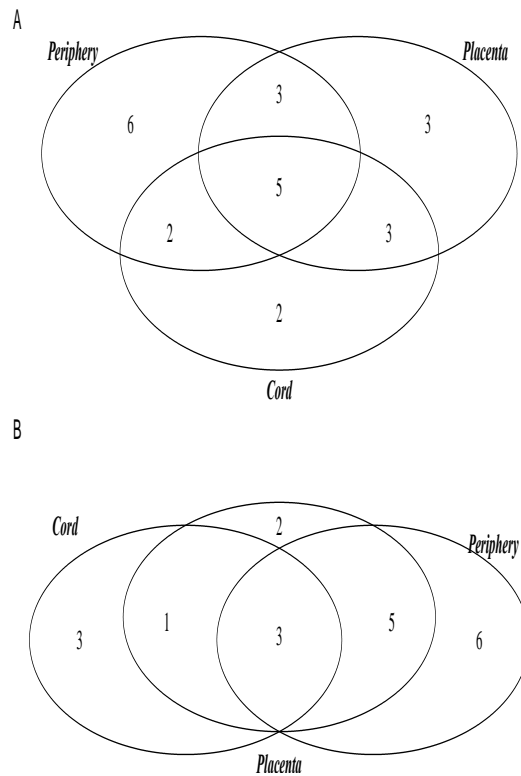
With regard to the genetic diversity, the distribution of different *MSP-2* alleles is shown in Table 3. A total, 30, 25 and 19 ( $P = 0.29$ ) alleles were detected respectively in the peripheral, placental and cord samples. The 3D7 family was predominant whatever sample

types. The mixed 3D7 and FC27 alleles were more prevalent in peripheral samples (26.3%) (Table 3).

**Table 3. Genetic diversity of *P. Falciparum* MSP-2 gene.** Total Nb. of alleles: total number of 3D7 and FC27 alleles of each compartment.

Alleles	Nb. of samples (%)			<i>P</i>	Fragment size range (bp)			Total Nb. of alleles (%)		
	Periphery	Placenta	Cord		Peripher y	Placenta	Cord	Periphery	Placenta	Cord
3D7	62 (54.4)	42 (60)	16 (45.7)	<0.001	180-680	140-600	120-480	16 (51.6)	14 (56)	12 (63.2)
FC27	22 (19.3)	20 (28.6)	11 (31.4)	0.14	260-620	260-540	240-520	14 (48.4)	11 (44)	7 (36.8)
3D7 + FC27	30 (26.3)	8 (11.4)	8 (22.9)	<0.001						
Total	114 (100)	70 (100)	35 (100)	<0.001				30 (100)	25 (100)	19 (100)

The 3D7 and FC27 alleles identified in the paired peripheral / placental, peripheral / cord and placental / cord blood samples were compared. The proportion of samples matched with identical and partially concordant alleles is shown in Figure 2, which summarizes the number of alleles found in peripheral, placental and cord blood. Some samples revealed the same alleles in the three compartments. In other cases, one or both samples had alleles not detected in the other ones.



**Fig. 2:** comparison of *P. falciparum* MSP-2 alleles detected in paired peripheral, placental and cord blood from Congolese women at delivery. (A) 3D7 family. (B) FC27 family.

### 3.4. Multiplicity of *P. falciparum* infections

The MOI was  $1.4 \pm 0.01$  (ranged 1-4 clones) in peripheral,  $1.2 \pm 0.01$  (range 1-3 clones) in placental and  $1.4 \pm 0.01$  (ranged 1-3 clones) in cord samples. Of 114 infected samples from peripheral blood, 110 (96.5%) contained single and 40 (35.1%) multiples infections. Of the 70 infected samples from placental blood, 46 (65.7%) harboured single and 24 (34.3%) multiples infections. In cord, 32 (91.4%) of 35 infected samples contained multiples infections and 3 (8.6%) samples have single infection. The MOI was neither affected by maternal age, nor gravidity or SP doses administered (Table 4).

**Table 4: Multiplicity of *P. falciparum* infections according to age of mother, gravidity and SP doses administered.** Prevalence of infection determined by PCR, including positive peripheral, placental and/or cord samples. Multiplicity of infections = mean number of *P. falciparum* genotypes per infected woman. The columns labelled *P* indicate the P-value of a mean comparison test, based on either the Wilcoxon test or Kruskal-Wallis test depending on the number of groups. The null hypotheses are explicated in the column header.

Multiplicity of <i>P. falciparum</i> infections (range) in the different compartments						
	Periphery	<i>P</i>	Placenta	<i>P</i>	Cord	<i>P</i>
Overall	1.4 (1-4)		1.2 (1-3)		1.4 (1-3)	
Age (year). Wilcoxon test with $H_0: m(< 18) = m(\geq 18)$ .						
< 18	1.4 (1-2)	0.79	1.3 (1-2)	0.78	2.0 (1-2)	0.88
$\geq 18$	1.4 (1-4)		1.2 (1-3)		1.4 (1-3)	
Gravidity. Wilcoxon test with $H_0: m(\text{Paucigravidae}) = m(\text{Multigravidae})$ .						
Paucigravidae	1.4 (1-3)	0.26	1.1 (1-3)	0.94	1.3 (1-3)	0.26
Multigravidae	1.3 (1-4)		1.3 (1-3)		1.4 (1-3)	
IPTp-SP. Kruskal-Wallis test with $H_0: m(\text{no dose}) = m(1 \text{ dose}) = m(2 \text{ doses}) = m(3 \text{ doses})$						
No	1.2 (1-2)	0.39	1.1 (1-2)	0.39	1.7 (1-3)	0.39
1 dose	1.4 (1-3)		1.2 (1-3)		1.7 (1-3)	
2 doses	1.6 (1-4)		1.1 (1-2)		1.6 (1-3)	
3 doses	1.1 (1-3)		1.3 (1-3)		1.1 (1-3)	

#### 4. Discussion

The current study is the first of its kind to evaluate the frequency of sub-microscopic *P. falciparum* infection among malaria asymptomatic Congolese women at delivery. The main findings of this study are the high prevalence of sub-microscopic infection at delivery, high diversity of parasites circulating in the population, and differential patterns of *MSP-2* allelic

families in matched peripheral, placental and umbilical cord parasite isolates from even the same individual.

The prevalence of sub-microscopic *P. falciparum* infection in the peripheral and placental blood was higher than microscopic infection in all compartments. In the cord compartment, the *P. falciparum* infection was found only by PCR. This indicates that the threshold of detection of *P. falciparum* by microscopy in pregnant women strongly under-estimates the actual infection rates (Francine et al., 2016; Mockenhaupt et al., 2000). Thus, reliance on microscopy may lead to miss diagnosis and failure to avert adverse effect of malaria during pregnancy (Mockenhaupt et al., 2000; Ntoumi et al., 2013; Ntoumi et al., 2016).

The prevalence of sub-microscopic infection was generally high at delivery. This suggests that pregnant women remained at increased risk of new infections during the late period of their pregnancies after receiving IPTp-SP, which is mostly administered during the second trimester. This increased danger may explain why despite the risk of infection being significantly reduced among delivered women who received 2 or 3 doses of IPTp-SP, there were still about 20% who harboured sub-microscopic infection. The proportion of sub-microscopic infection in our study is quite small compared to those reported from Gabon (Tshibola et al., 2014). This difference might be due to the transmission intensity of the parasite at the different study sites and possibly differential host factors such as a degree of immunity that might restrict parasites growth to microscopically undetectable levels. Alternatively, these low-density infections may represent recently acquired infections, which could reach detectable levels by microscopy if infections were not treated.

All microscopic placental blood infections were microscopically detectable in the peripheral blood. However, 42 women with sub-microscopic infection of the placental blood showed no evidence of peripheral blood infection. During pregnancy, parasites expressing a ligand for chondroitin sulphate A tend to adhere to placenta and sequester in this unique compartment (Fried and Duffy, 1996) and are rarely detected in the peripheral blood. This might have accounted for the observation made in these 42 participants. The prevalence of placental *P. falciparum* infection was lower than peripheral, which is consistent with some of the previous reports (Omalu et al., 2012; Hviid et al., 2011) but not with others

(Mayengue et al., 2004; Ouédraogo et al., 2012). The high prevalence of infection in peripheral than placenta blood may explain the new *P. falciparum* infections in third trimester of pregnancy.

Asymptomatic *P. falciparum* infection is highly prevalent in malaria endemic regions, which raises a serious concern about the efforts at eliminating the parasite (Riley et al., 2001). Particularly, sub-patent malaria is still transmissible and will complicate the elimination of malaria in high-transmission regions (Sturrock et al., 2013). Most studies suggested that exposed individuals are likely to carry chronic asymptomatic *P. falciparum* infections (Sturrock et al., 2013; Ouédraogo et al., 2016), a situation in which the majority of this population can act as a reservoir for malaria transmission.

Our results showed 15 women with sub-microscopic infection in the cord samples with no evidence of infection in peripheral and/or placental samples. The unique presence of certain parasites in the cord can be explained by parasite clearance in the placenta and / or maternal-foetal transfusions before or during birth (Riley et al. 2001).

The *MSP-2* marker is very polymorphic. We detected 24 distinct 3D7 alleles and 20 distinct FC27 alleles. Similar results were observed in Gabon (Kassberger et al., 2002, Mayengue et al., 2004) in Senegal (Schleiermacher et al., 2001). The 3D7/*MSP2* allele was predominant such as reported in the previous report in the same site (Francine et al., 2016). Similar results are observed in Senegal (Jafari-Guemouri et al., 2005), Sudan (Fadlelseed et al., 2017). In Gabon, the predominant allele was FC27/*MSP2* (Kassberger et al., 2002, Mayengue et al., 2004). Allelic frequencies are very different from one family to another and the distribution of these alleles reveals the genetic variability present in the parasite population and might reflect the geographical variation of parasite genotype and the level of transmission.

Multi-clonal infection was detected respectively in 40 (35.1%), 24 (34.3%) and 32 (91.4%) of the peripheral, placental and cord samples. These results are the characteristic of the stable malaria transmission area (Mayengue et al., 2004).

The MOI could be used in the evaluation of malaria interventions as it is an indicator of

malaria infection in humans and may, to some extent, reflect transmission and immunity malaria (Smith et al., 1999). In this cohort the MOI is lower than that reported (about 1.6 MOI) in the previous study in the same site (Francine et al., 2016) and in other malaria endemic area (Beck et al., 2001; Walker-Abbey et al., 2005). Furthermore, The low MOI could be explained by the use of impregnated mosquito nets and the degree of immunity. As a result, the low parasite density of this study, which results in low MOI, can be observed, but this did not affect the diversity of the parasites in circulation (Ibara-Okabande et al., 2012).

The high prevalence of asymptomatic malaria in young pregnant women and those in their first and second pregnancies than those in their third or more pregnancies have reported (Smith et al., 1999). With successive pregnancies, women acquire a gravidity-dependent anti-malarial immunity, resulting in a decrease in both prevalence and severity of infection (Smith et al., 1999). Studies have demonstrated a correlation between the MOI and parasite density as well as age of the study participants (Mayengue et al., 2004; Omer et al., 2011). In our study, MOI was associated with neither maternal age nor gravidity, which is in line with other study (Kassberger et al., 2002, Walker-Abbey et al., 2005). Age and the acquisition of pregnancy-related immunity do not appear to result in elimination of parasites or reduction of the number of different infecting strains. The SP doses have not reduced the MOI. This might result from the carriage of SP-resistant parasites in such women as suggested earlier (Moussiliou et al., 2013, Koukouikila-Koussounda et al., 2015; Ojurongbe et al., 2018).

The cross-sectional nature of the study did not allow participants to be followed until delivery. The lack of current entomological data during the collection of samples and the lack of data on the use of mosquito nets by pregnant women do not allow us to deepen the analyses on the low multiplicity of infections.

## **5. Conclusions**

In summary, this study showed that there is a high prevalence of sub-microscopic infection and a moderate genetic diversity of *Plasmodium falciparum*. Age, gravidity and doses of preventive treatment based on sulfadoxine-pyrimethamine do not interfere with the



multiplicity of infections. These results contribute to the understanding of the dynamic of parasites in blood sample types during pregnancy and parasite population circulating in Congo.

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## **Competing interests**

The authors declare that they have no competing interests.

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## **Data availability**

Raw data of the work presented here are available upon request.

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