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ORIGINAL PAPER

F. Kassberger · A. Birkenmaier · A. Khattab
P.G. Kremsner · M.-Q. Klinkert

PCR typing of *Plasmodium falciparum* in matched peripheral, placental and umbilical cord blood

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Abstract A study was carried out in Lambaréné, Gabon, to analyse malarial infections in pregnant women. Blood samples from peripheral circulation and term placentas from 37 women were diagnosed as parasite-positive by thick blood smears. Infection was confirmed by PCR, using single-copy merozoite surface protein 1 and 2 genes. Of the 37 matched cord blood samples, 17 were positive by PCR amplification, even though all but one were microscopically negative. Five of these 17 samples were verified as positive in an antigen detection assay for histidine-rich protein 2. A comparison of alleles from these compartments indicates that single clonal infections were predominant for the cord samples, while double and triple infections were more common for peripheral and placental samples. Knowledge of the occurrence of parasites in each blood sample type is important towards understanding population dynamics in pregnant women and the development of immunity in infants to selected genotypes.

Introduction

In disease-endemic areas, multiple and continued exposure to malarial infections persists throughout life. A certain degree of immunity is acquired in the first decade of life, characterised by a gradual decrease in the frequency and severity of malaria (Forsyth et al. 1989; Marsh et al. 1989; Kremsner et al. 1995; Bull et al. 1998). Thus, the vast majority of deaths and severe malaria morbidity in young children occur before anti-malarial immunity develops (McGregor et al. 1983; McGregor 1984; Snow et al. 1999; Steketee et al 2001). However, during pregnancy and especially the first pregnancy, this immunity is diminished, with the result that women become more frequently infected and parasite densities in the placenta can be high. Complications from placental infections are associated both with maternal anaemia, maternal, perinatal and infant mortality and with intrauterine growth retardation and low birth weight. Such deleterious effects become less frequent as the parity of the women increases (Brabin 1983).

Since malaria in the infant is rare, it is widely believed that the placenta acts as an important barrier and prevents malaria parasites from passing from the maternal to the foetal circulation. Malarial infections in the newborn are rare, even in areas where disease transmission is intense and varies between sites and where placenta parasitaemias are very high; and this is supported by a report from an area with high transmission showing that parasites are rarely transmitted across the placenta (Adachi et al. 2000). However, there are other studies documenting foetal malarial infections as a result of malaria during pregnancy (Lehner and Andrews 1988; Larkin and Thuma 1991; Fischer 1997). The most extensive of these studies included 2,080 women participating in a trial of malaria prophylaxis during pregnancy in Malawi (Redd et al. 1996). From the 388 placentas which carried *Plasmodium falciparum* parasites, 140 of the samples were reported to have umbilical cord parasitaemia.

In order to examine the situation in Lambaréné, Gabon, which is hyperendemic for malaria and where transmission is perennial (Wildling et al. 1995; Sylla et al. 2000; Planche et al. 2001), we collected blood samples from term placentas and from matched peripheral and umbilical cord blood samples from women delivering

F. Kassberger and A. Birkenmaier contributed equally to this work

F. Kassberger · A. Birkenmaier · A. Khattab
P.G. Kremsner · M.-Q. Klinkert (✉)
Department of Parasitology,
Institute for Tropical Medicine,
University of Tübingen, Wilhelmstrasse 27,
72074 Tübingen, Germany
E-mail: mo.klinkert@uni-tuebingen.de
Tel.: +49-7071-2980240
Fax: + 49-7071-295189

F. Kassberger · P.G. Kremsner
Medical Research Unit,
Albert Schweitzer Hospital, Lambaréné, Gabon

at the Albert Schweitzer Hospital. Samples from all three compartments were analysed for the presence of *P. falciparum* parasites, in order to examine malarial infection and transmission in pregnant women. A PCR-based typing strategy amplifying two highly polymorphic marker genes encoding merozoite surface antigens 1 (*msp1*) and 2 (*msp2*) was used (Contamin et al. 1995; Ntoumi et al. 1995). An early infection-detection marker may be clinically relevant and important in our understanding of disease development and immunity in infants at risk of malaria.

Materials and methods

Study design

The study was carried out at the Albert Schweitzer Hospital in Lambaréné, Gabon. Study participants were those who presented at the maternity ward of the hospital for delivery, from whom written informed consent was obtained. A total of 273 women was initially recruited in our study and subsequently 37 samples were further analysed. These samples were matched for three compartments, namely peripheral, placental and cord blood and were chosen on the basis that both peripheral and placental blood tested *Plasmodium falciparum* positive in their thick blood smears. The few rare cases where only one of the compartments was infected were omitted from this study. On admission (whenever possible), demographic data (e.g. ethnic group, date of birth, weight, history prior to admission) were collected. Hospital records showed that all women were examined and blood smear positivities were detected at different time-points during their pregnancies. Those with clinical symptoms of malaria (15 out of 37) were treated with quinine. Despite treatment, all these patients had parasites in the placentas at delivery, regardless of the time of infection during the pregnancy. Only four of the 37 study participants did not take malaria prophylaxis throughout their pregnancies. The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital.

Blood samples

Peripheral blood was collected in a monovette. Parasite densities were determined in peripheral blood by the conventional microscopic examination of Giemsa-stained thick blood smears. Slides were examined using a light microscope covering 100 fields with the 100× objective under oil immersion; and parasite densities were recorded using the Lambaréné method (Planche et al. 2001). In addition to performing thick blood smears, peripheral blood samples were also centrifuged to separate the pellet containing the packed erythrocytes from the plasma. Packed erythrocytes (100 µl aliquots) were stored at –80 °C and subsequently used for DNA preparation, PCR amplification and genotyping experiments. Placental blood was extracted from placentas by squeezing a piece of placenta tissue and leaving it to drain into a solution containing 10 units of heparin/ml dissolved in 0.9% NaCl. Placental blood was examined for the presence of malaria parasites and packed erythrocytes from the blood were collected by centrifugation and aliquoted into 100 µl portions. In addition, cord blood was taken from the umbilical vein using a monovette and systematically analysed using standard thick blood films, as described above. Packed erythrocyte pellets of cord blood samples were prepared, as above.

Parasite DNA preparation and PCR amplification

DNA was extracted from 100 µl of packed erythrocyte pellets from the periphery, placenta and umbilical cord using the QIAamp DNA blood mini kit (Qiagen). PCR genotyping was performed on DNA prepared from the equivalent of 2–5 µl of the packed erythrocyte pellet, using well established single and nested PCR procedures, as described by Contamin et al. (1995), Ntoumi et al. (1995), and Zwetyenga et al. (1998). The surface antigens MSP-1 and MSP-2, which served as targets in the PCR amplification, belong to genes highly conserved for the most part of their sequences, except for the presence of a region of repeats within the genes.

Primers used for the *msp1* gene were T5 and T6 and covered the conserved flanking regions of the block 2 repeat region. Block 2 is particularly polymorphic and three distinct families, K1, MAD20 and RO33, have been described. In the nested PCR amplifications that followed, the K1 family was obtained using K1 as the forward primer and K2 as the reverse primer. MAD20-specific sequences were amplified with forward M1 and reverse M2. RO33 alleles were obtained using R1 and R2. Primers 1 and 4 for the *msp2* family covered the conserved regions flanking the central variable region. This polymorphic region contains unique sequences characteristic for each of the two distinct families, FC27 and 3D7. Using specific FC1 and FC2 primers, the FC27 family of genes were amplified. Similarly, D1 and D2 served as specific 3D7 primers. Each set of nested primers specific for the different allelic families yielded products of varying lengths, depending on the nature of the isolates in terms of their polymorphic domains and repeated regions. The amplified PCR products were loaded onto 1.5% agarose gel (SeaKem, Sigma), subjected to electrophoresis, stained with ethidium bromide and visualised under ultraviolet light. Size polymorphism was analysed in each allelic family, based on the assumption that one band represented one amplified PCR fragment derived from a single copy of the *P. falciparum msp-1* or *msp-2* gene.

Previously established DNA positive control samples used were strains K1, 7G8 and HB3 clones for K1, MAD20 and RO33 MSP-1 allelic family detection, respectively. Expected band sizes were 200 bp for the K1 allele, 220 bp for MAD20 and 150 bp for RO33. Positive controls for the FC27 and 3D7 MSP-2 types were taken from K1 and V1/S DNA, respectively, and the corresponding bands were 400 bp and 280 bp in length. In all PCR reactions, a negative control sample with no template DNA were carried out in parallel.

Detection of *P. falciparum* antigens

An immunochromatographic assay to detect *P. falciparum* antigens in sera collected from peripheral, placental and cord blood was performed using a commercially available reagent (RIDA MalaQuick Kombi, R-Biopharm, Darmstadt, Germany). The test assay was carried out according to the manufacturer's instructions and is based on the detection of the histidine-rich protein of the *P. falciparum* parasite (PfHRP-2), capable of interacting with specific anti-PfHRP-2 antibodies immobilised on the test strip.

Statistical analysis

Our data were analysed by the Pearson 2×2 χ^2 test, Fisher's exact test, Spearman's rank correlation coefficient test and the Wilcoxon/Kruskal-Wallis non-parametric test.

Results

Determination of parasitaemia by thick blood smear

From our study, 37 of the 273 samples collected were chosen for analysis on the basis of *Plasmodium falciparum* thick blood smear positive slides both in peripheral and placental compartments. In order to analyse more closely the phenomenon of malarial infections of neonates via transmission across the placenta, matched

umbilical cord blood samples from these participants were also examined in thick blood smears. All cord slides were found to be negative, with the exception of one case. Since this was a stillbirth, the most likely explanation for umbilical cord blood parasitaemia is the breakdown of the maternal–foetal barrier in controlling transmission of parasites to the foetus.

Msp1 genotyping by PCR amplification

Nested and semi-nested PCR amplifications for the presence of the *msp1* gene (block 2), as previously described, were also carried out. *P. falciparum* parasites carried multiple *msp1* alleles belonging to the K1, MAD20 and RO33 families. As expected, results obtained by the PCR technology confirmed the thick blood smear detection of *P. falciparum* in the peripheral and placental samples. An example of a typical PCR experiment performed on DNA prepared from all three compartments from one patient is shown in Fig. 1A–C. The PCR products using DNA from reference *P. falciparum* strains are shown in Fig. 1D. The PCR detection of *P. falciparum* in cord blood samples was rather surprising and is in contrast to our microscopic findings. In 15 of the 37 samples, at least one of the *msp1* markers was detected.

The comparison of *msp1* alleles in the matched placental, peripheral and umbilical cord samples is summarised in Fig. 2. The fact that a large number of different *msp1*-specific bands was observed in all three compartments is taken as an indication of the existence of clonal diversity in the study population. The proportions of each of the three individual *msp1*-specific alleles, K1, MAD20 and RO33, were fairly well distributed in the peripheral and placental parasite populations. In the cord positive samples, however, the ratio of K1 variants compared to non-K1 variants are higher than in the comparison made for the peripheral PCR-positive samples (χ^2 test, $P=0.01$). Additionally, more K1 genotypes than the other two genotypes were found in the cord samples than in the placental PCR-positive samples ($P=0.05$). Overall, the K1 variant is the most prevalent variant in our study population, as previously demonstrated (Kun et al. 1998).

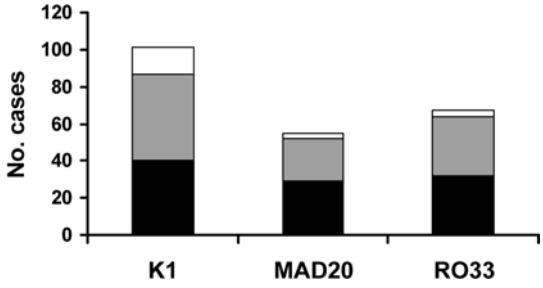
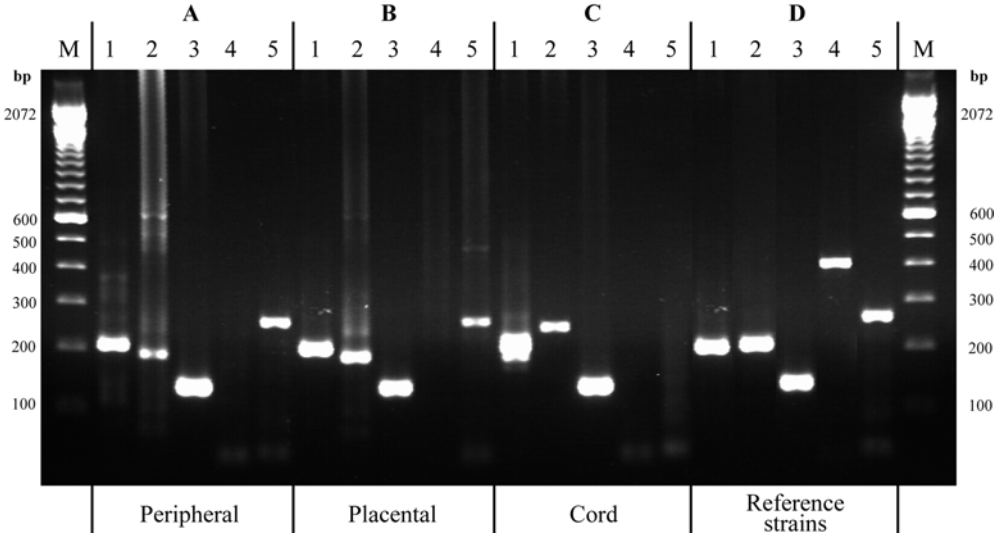


Fig. 2. Comparison of the *msp1* alleles in the matched peripheral, placental and cord blood samples. All three alleles were represented in similar proportions in the peripheral (*black*) and placental (*grey*) samples. However, in the cord blood (*white*) the K1 allele was present in a higher proportion than the RO33 and MAD20 alleles taken together, when compared with the peripheral ($P=0.01$)

Msp2 genotyping by PCR amplification

With respect to the *msp2* gene, PCR amplification carried out on specific sets of primers allowed the identification of both FC27 and 3D7 allelic families. In the placenta and in the periphery, several distinct forms of FC27 and 3D7 were observed and the distribution of these two alleles in these compartments was not significantly different. However, the distribution of the FC27 genotype in the

Fig. 1. Agarose gel analysis of the amplification reactions. The PCR products were obtained from genomic DNA prepared from: *A* peripheral blood, *B* placental blood, *C* cord blood and *D* positive *Plasmodium falciparum* clones used as references for the amplification reaction. Size polymorphisms for the *msp1* and *msp2* loci were analysed in the following order: *1 msp1* K1, *2 msp1* MAD20, *3 msp1* RO33, *4 msp2* FC27 and *5 msp2* 3D7. *M* Size markers



cord blood was found more frequently than 3D7 when compared with the FC27 to 3D7 ratio of our peripheral ($P = 0.05$) and placental ($P = 0.05$) samples (Fisher's exact test; Fig. 3). An example of the PCR products as amplified from all three compartments of one patient and the reference strains are shown in Fig. 1.

Genotyping based on the *msp2* marker appears to be less sensitive than *msp1*. It was observed that only seven of the 37 cord blood samples tested positive in PCR amplifications using *msp2*-specific primers, in contrast to the 15 samples that were positive with the *msp1* primers. Possible explanations for this insensitivity of the *msp2* marker could include low DNA concentrations in the samples or the presence of point mutations at the positions where the *msp2*-specific primers anneal. Interestingly however, two of the *msp2*-positive samples were *msp1*-negative. Taken together, our results of both the *msp1* and the *msp2* markers, 17 of the 37 cord blood samples (46%) were found to be positive.

Comparison of alleles between parasites in the different compartments

The number of *msp1*alleles observed in this population both in peripheral and placental blood ranged over 1.0–5.0, with a mean of 2.7 ± 1.2 for peripheral samples and a mean of 2.7 ± 0.9 for the placentas. Most of the individuals simultaneously carried at least one or two identical *msp1* alleles. In contrast, when compared with the presence of *msp2* alleles, 1.0–4.0 different *msp2* alleles in both compartments were readily distinguished. In the periphery, a mean of 1.4 ± 0.7 *msp2* alleles was calculated and a mean of 1.6 ± 0.9 was found for the placenta. In general, there was good correlation in the number of infections in the matched placental and peripheral blood samples (Spearman's rank test, $r = 0.4$, $P < 0.01$).

To address the question regarding the relationship between placental and peripheral parasites, *msp1* and *msp2* genotype compositions and band patterns of these parasite populations were compared with each other (Table 1). The genotype compositions of parasites in the placenta and in the peripheral circulation were not always identical, but in fact were overlapping in their pattern, in that they shared common genotypes and were divergent from each other. When genotype profiles of the placental and cord blood parasites were compared, similar observations were also made.

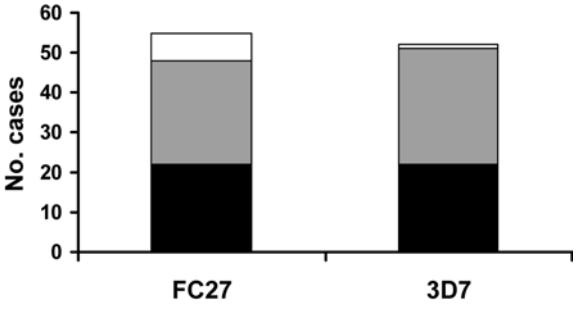


Fig. 3. Comparison of the *msp2* alleles in the matched peripheral, placental and cord blood samples. Both FC27 and 3D7 alleles were well distributed in both the peripheral (*black*) and placental (*grey*) compartments. In the cord blood (*white*), FC27 were present in a higher proportion than 3D7, the comparison with both the periphery and the placenta being significant ($P = 0.05$)

Detection of *P. falciparum* antigens

In the peripheral samples, the positivity of PfHRP-2 antigen was fairly consistent with the results of the thick blood smear. Only two thick blood smear positive cases tested negative for the antigen. In comparison, samples from the placenta showed consistency between thick blood smear and antigen test, demonstrating the reliability of the method and confirming the results of other workers (Funk et al. 1999; Jelinek et al. 2001). However, when the antigen test was applied to the 37 cord blood samples, in only five of the 17 PCR-positive cord blood samples was the PfHRP-2 antigen detected.

Multiplicity of infection

The number of alleles that were found in our genotyping experiments was used as a basis for calculating the multiplicity of infection, and the highest number of alleles (as represented by the number of PCR bands) visualised as either *msp1* or *msp2* PCR amplifiers was taken as the number of clones in the sample. A comparison of the parasite *msp1* and *msp2* genotypes present in the three compartments was performed (Fig. 4). A most noteworthy finding is that cord blood samples carried predominantly single clonal infections (Fisher's exact test, $P < 0.001$). In contrast, the peripheral blood samples of the majority of patients mostly carried double infections, whereas placentas to a great extent carried double or triple infections. The K1 genotype was observed to be the most common genotype found in cord blood. Multiplicity of infection did not depend on the age of the mothers, nor did it correlate with the

Table 1. Comparison of *msp1* and *msp2* genotype compositions between matched peripheral, placental and cord blood samples. While band patterns described as identical and divergent are self-explanatory, the overlapping pattern is considered by the presence of some alleles being common to parasites of both compartments

	Peripheral versus placental samples		Placental versus cord samples	
	Msp1 genotype ($n = 37$)	*Msp2* genotype ($n = 35$)	*Msp1* genotype ($n = 15$)	*Msp2* genotype ($n = 7$)
Identical	4	14	1	1
Overlapping	26	10	7	2
Divergent	7	11	7	4

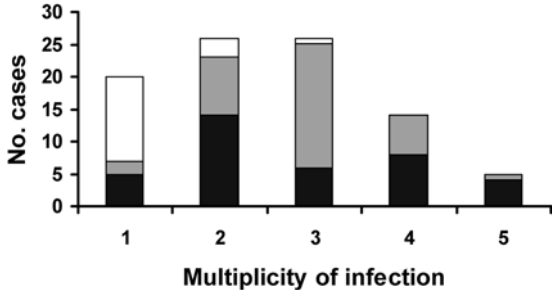


Fig. 4. Results of the combined *msp1* and *msp2* genotyping data. The number of women with different multiplicity of infections ranging over 1–5 in the peripheral (*black*), placental (*grey*) and cord (*white*) blood were counted. Most of the cord blood samples were infected with single clones

parasite densities that had been determined in the participants.

Parasite densities in the infected individuals were wide-ranging: peripheral parasitaemia were detected at 6×10^2–1.3×10^5 parasites/μl and placental parasitaemia differed over 2×10^3–1.25×10^6 parasites/μl. There was a significant correlation between the two (Spearman's rank test, $P<0.001$).

Analysis of parity

Parity in our study cohort ranged from one to seven. In our group of 37 women, we were able to classify 26 as paucigravidae (first two pregnancies) and 11 as multigravidae (at least third pregnancy). By comparing the number of total *msp1* and *msp2* clones in the peripheral compartment of the pauci- with the multigravid group, there was no significant difference in both groups of individuals (Wilcoxon/Kruskal-Wallis test). The paucigravid individuals also were not found to carry more placental infections than those in the multigravid group.

Discussion

A comparative analysis was carried out at the Albert Schweitzer Hospital in Lambaréné, Gabon, to examine the presence of *Plasmodium falciparum* parasites in the peripheral blood of the mother, the placenta and the umbilical cord. Women who were delivering at this hospital were recruited in our study ($n=273$) and were initially screened for malarial infections by thick blood smear. Next, 37 women were chosen for further study, based on the availability of blood samples from all three compartments. *P. falciparum* infections in whole blood and in the placentas of the subjects were confirmed using DNA prepared from packed erythrocytes for PCR amplification of the *msp1* and *msp2* genes.

At the time of sampling, the women had *P. falciparum* infections in both their peripheral and placental compartments. From genotyping experiments, we found that the populations of peripheral parasites are not always the same as those in the placenta and, in fact, some genotypes are detected only in one or the other compartment. The differences between parasite populations in peripheral blood and placenta may be explained by the extensive daily dynamics, whereby parasites present in the blood can differ from day to day (Färnert et al. 1997). The occurrence of different parasites at different time-points has also been observed in asymptomatic individuals.

The current hypothesis is that only those parasitised erythrocytes expressing distinct placental-binding membrane protein on the surface can be selected to cytoadhere to the placenta and subsequently cause disease. Moreover, the parasites selected to bind the placenta may multiply within the placenta as non-circulating parasites, as predicted by the ring-stage sequestration theory (Pouvelle et al. 2000). It is still not known whether and what proportion of parasitised erythrocytes from an infected placenta can again enter the circulation, but possibly the parasites circulating in the mother on consecutive days may be the same as those detected in the placenta. The factors controlling the process remain to be investigated. Preliminary data from microsatellite typing experiments indicate that the parasites present in the placenta do not constitute a specific subpopulation (unpublished data).

As expected, almost all of the parasite genotypes present in the cord were also seen in the corresponding placenta. However, in some very rare cases the genotypes do not appear to be related to each other. Possibly, a different dominant genotype has taken over in the placental tissue before the parasites that do cross the placental barrier become eliminated or suppressed.

A most notable observation is the high number of PCR-positive umbilical cord samples. PCR examination of our matched thick blood smear positive samples showed that 46% were cord blood positive. From our genotyping experiments, the proportion of *msp1* K1 and *msp2* FC27 genotypes was higher for cord blood samples than the other alleles when compared with the other two compartments. Most significantly, we found only one variant clone in a high proportion of our cord blood samples, in contrast to several mixed variants in the peripheral and placental blood samples.

The significance of cord blood positivities is not yet clear. The placental barrier normally prevents transplacental passage of *P. falciparum* parasites. However, under certain circumstances, for example in the case of the stillbirth, the maternal–foetal interface may be impaired in its function to control parasite transmission to the foetus. If mother-to-foetus transmission of *P. falciparum* during a placental malaria infection is more frequent than reported, the immediate question to ask is why parasites are not more commonly detected in cord blood smears. In order to account for negative thick blood smears and positive PCR samples, we can only speculate that parasite numbers are too low to be detected. The next logical question to ask is why

transplacental passage of parasites does not normally cause illness in the new-born. Some arguments to explain the absence of malaria in the new-born include the failure of parasites to grow in cord blood and fast elimination of parasites from the foetal circulation (Pasvol et al. 1976, 1978; Shear at al. 1998). An alternative explanation for PCR positivity in cord blood samples is to consider that parasite macromolecules and not live parasites have crossed the placental membrane. Recently, reports based on the very sensitive real-time quantitative PCR assay demonstrated free circulating nucleic acids which originated from a number of infectious agents in human plasma and serum (Jacobsen et al. 1998; Fleishhacker 2001). Of relevant interest to this study is the detection of circulating *P. falciparum* DNA and antigen, not only in samples with a positive blood smear, but also in microscopy-negative cases.

In order to determine the incidence and presentation of pregnancy-associated malaria, both in the mother and the new-born, it is important to study *P. falciparum* infections not only in the circulation but also in the placenta and in the umbilical cord, as these tissues become available. Our present study represents a first step to gain an insight into *P. falciparum* pathogenesis in relation to the transmission patterns within the study area and provides us with a basis to investigate whether and how maternal infections influence parasite development and disease progression in a new-born.

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