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INCREASED MULTIPLICITY OF *PLASMODIUM FALCIPARUM* INFECTIONS AND SKEWED DISTRIBUTION OF INDIVIDUAL *MSP1* AND *MSP2* ALLELES DURING PREGNANCY IN NDIOP, A SENEGALESE VILLAGE WITH SEASONAL, MESOENDEMIC MALARIA

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Abstract. Pregnancy is associated with a greater susceptibility to *Plasmodium falciparum* infections, which may result in serious complications affecting both the mother and the fetus. To compare allelic diversity and multiplicity of infection in the same women during and outside pregnancy, we conducted a retrospective analysis of the monthly fingerprick blood samples collected during a longitudinal survey conducted in Ndiop, a Senegalese village with mesoendemic malaria. Merozoite surface protein-1 (*msp1*) block 2 and merozoite surface protein-2 (*msp2*) genotypes were determined for 308 blood samples collected from 20 women. Pregnancy was associated with a significantly higher prevalence of *P. falciparum* infection, higher parasite densities, and a higher multiplicity of infection. The highest multiplicity of infection was observed in the youngest pregnant women. Because of co-linearity, it was not possible to dissociate the impact of age from that of parity on multiplicity of infection. Some individual *msp1* and *msp2* alleles showed a highly skewed pregnancy-associated distribution. These results indicate that pregnancy is associated with increased permissiveness to a large number of clones, as well as with infection by specific genotypes.

INTRODUCTION

Pregnancy is associated with a greater susceptibility to *Plasmodium falciparum* infections.[1] Depending on the level of immunity before pregnancy, an increased frequency and severity of maternal infection may be observed during pregnancy.[2] In endemic areas with intense malaria transmission, where women have acquired a strong protective immunity prior to pregnancy, primigravidae are more affected,[1,3] but multigravidae are also at increased risk for malaria.[4] In mesoendemic areas, susceptibility to *P. falciparum* infections in secundigravidae may resemble the susceptibility observed in primigravidae living in high transmission areas.[2] In areas of low transmission, pregnant women are at increased risk for severe disease. The increased maternal susceptibility to malaria has adverse consequences for the fetus, with premature delivery, reduced birthweight, and in some cases abortion and stillbirth.[2]

In recent years, two factors contributing to the higher susceptibility to *P. falciparum* infections during pregnancy have been considered: 1) the transient pregnancy-associated depression of cell-mediated immunity[5,6] and 2) the accumulation of parasites in the placenta due to adhesion of infected red blood cells expressing specific variant antigens to particular receptors on the placental lining.[7,8] Primigravidae are believed to be more susceptible to infection since they have not yet encountered these variant types and consequently have no acquired specific immunity. However, little is currently known about the number of distinct infections experienced by the mother throughout pregnancy and about the multiplicity and diversity of infections during pregnancy. The work reported here was undertaken to address these issues.

The epidemiological studies conducted in Ndiop, a Senegalese village where the entire population has been involved in a longitudinal prospective malaria epidemiology study since 1993, provided the possibility of investigating *P. falciparum* infections during pregnancy and of comparing them with those occurring outside pregnancy in the same women.[9,10] In this village, transmission is moderate and highly seasonal, and malaria patterns are typical of mesoendemic areas.[10] We conducted a retrospective analysis of *P. falciparum* infections using blood samples collected as part of the systematic monthly blood sampling of village residents who agreed to participate. We analyzed samples collected before, during, and after pregnancy from women who became pregnant and delivered since the commencement of the epidemiological survey. *P. falciparum* infections were investigated using polymerase chain reaction (PCR) to detect allelic polymorphism of two single-copy genes, the merozoite surface protein-1 (*msp1)* (block 2) and merozoite surface protein-2 (*msp2) (*central domain) genes. This allowed detection of genetically distinct parasites and estimation of the minimal number of co-infecting parasites present in a blood sample.[11–14] We determined the multiplicity of *P. falciparum* infections in primigravidae, secundigravidae, and multigravidae and compared the multiplicity of infection and allelic distribution before, during, and after pregnancy.

MATERIALS AND METHODS

Study site and blood collection. Ndiop, a village of about 350 inhabitants, is located in Sine-Saloum, a Sahelo-Soudanian region of Senegal where a longitudinal epidemiological and entomologic follow-up has been conducted since 1993.[9,10] The annual entomologic inoculation rate varies between 7–63 infective bites/person/year. Monthly longitudinal records of the entomologic rate are available. Malaria transmission is highly seasonal. No transmission is detected in the dry season which usually lasts 7–8 months.[9] The longitudinal follow-up includes daily clinical surveillance by a medical team living in the village. Detailed data on clinical

episodes and treatment(s) administered are available for every individual enrolled in the study. None of the women studied here took antimalarial chemoprophylaxis during or outside pregnancy. In pregnant women, all cases of fever associated with a positive blood smear, whatever the parasite density, were considered possible malaria attacks and presumptively treated with antimalarials. The Ministry of Health (Senegal), the ethical committee, and the Conseil de Perfectionnement of the Pasteur Institute of Dakar approved the study protocols.

Monthly fingerprick samples have been collected from all permanent residents of Ndiop after informed consent of the individuals or their parents since 1993. The 308 blood samples studied here were obtained from 20 women in the course of 23 pregnancies and from the same women the year before and/or after pregnancy. Because the exact time of conception is unknown, we defined the duration of pregnancy as 9 months preceding the birth. This is somewhat arbitrary since malaria is frequently associated with premature delivery in Africa.[1,2] All blood samples were collected and stored as described.[15] Blood samples obtained during and/or four days after a clinical episode were excluded from the present study. Antimalarial treatment in the 30 days preceding the date of blood sampling was recorded for only five blood samples.

Parasite density was determined as described.[15] Sickle cell trait and glucose-6-phosphate-dehydrogenase (G6PD) deficiency were diagnosed in one and three women, respectively. The majority of the women did not use bed nets; five women used bed nets on an irregular basis. Approximately two-thirds of the women had lived at least two of the last three years in the village.

Extraction of DNA and PCR genotyping. Extraction of DNA was carried out as described[16] and genotyping accomplished by nested PCR. For *msp1* block 2 typing, primers A+B[13] were used for the primary PCR, followed by family-specific primers K1+K2, M1+M2, and R1+R2[16] for individual family-specific nested reactions. For *msp2* typing, the primary PCR was done using the conserved primers 2+3[13] flanking the central polymorphic domain, and the family-specific PCR using primers A1+A2 and B1+B2[12] were subsequently used for the nested family-specific reactions. The reaction conditions were as described by Zwetyenga and others.[16] Amplifications were done using a Hybaid thermal reactor (Cera-Labo, Ecquevilly, France). Both primary PCRs were carried out with 2.5 μl of DNA (equivalent to 5 μl blood), which was denatured in the PCR mixture at 94°C for 5 min, followed by 30 amplification cycles (2 sec at 94°C, 1.5 min at 57°C, and 2 min at 72°C). The nested family-specific PCRs were carried out with 1 μl of the primary PCR sample. After denaturing at 94°C for 5 minutes, *msp1* amplification was done for 30 cycles (2 sec at 94°C; 1.5 min at 65°C for K1+K2, 67°C for M1+M2, 68°C for R1+R2, and 2 min at 72°C). Amplification of *msp2* was done for 25 cycles (2 sec at 94°C; 1.5 min at 58°C for A1+A2 or B1+B2, and 2 min at 72°C). The primary PCR products and secondary *msp2* PCR products were analyzed for size polymorphism on 2.5% NuSieve® GTG® agarose gels (FMC Bioproducts, Tebu, Le Perray-en-Yvelines, France). The secondary *msp1* PCR products were separated on 3% Metaphor® agarose gels (FMC Bioproducts). The DNA was visualized under ultraviolet light after staining with 0.5 μg/ml ethidium bromide. Fragment size was calculated relative to the molecular size standards (100 and 20 base pair [bp] ladders [Advanced Biotechnologies Ltd., Epsom, UK]). The discrimination level for distinct alleles was considered to be 10 base pairs, and distinct alleles were consequently assigned to 10 base-pair bins.

Distribution of allelic families, multiplicity of infection, and statistical analysis. PCR fragments were assigned to specific allelic families according to secondary PCR results. Multiplicity of infection was calculated by combining *msp1* and *msp2* PCR genotyping. The highest number of bands detected, whatever the locus, was used as the value for overall multiplicity of infection of the sample.

Prevalence rates of positive nested-PCR blood samples, prevalence of each allelic family, prevalence of alleles observed in ≥ 10 samples, and multiplicity of infection were analyzed using a generalized estimating-equation approach that allows analysis of repeated measures. This can be implemented for either logistic or Poisson responses using the SPIDA statistical package (SPIDA Version 6; Statistical Computing Laboratory, Eastwood, New South Wales, Australia).[17] We used an exchangeable correlation structure where the correlation between observations made in the same person at different time points is assumed to be identical. With this model, the estimated odds ratio can be considered to be an estimate of individual relative risk (of being positive or of having an additional band).[18] The differences were tested by the Wald test.[19] The explanatory variables, namely age, parity, active transmission, antimalarial treatment, and pregnancy, were first included together in the models and subsequently removed if their effect was not significant. Their interaction terms were also tested. Transmission was considered individually for each blood sample by determining presence or absence of transmission as recorded in the entomologic follow-up in the village during the 60 days preceding the date of blood sampling.

Overall comparisons of the distribution of alleles from *msp1* or *msp2* loci were tested using the methods described by Fleiss and O'Quigley,[20,21] which allow the comparison of several proportions in the presence of low cell expectations under the assumption of independence of observations made in the same person at different time points. This assumption was acceptable in the present data set. Comparison of the prevalence of alleles detected < 10 times was analyzed by Fisher's exact test.[22] For analysis of individual alleles detected in ≥ 10 samples, the significance cut-off was calculated using the correction of Fisher,[22] namely 5/n percent where n is the number of comparisons. For *msp1* (n = 13) and *msp2* (n = 16), this value was 0.0038 and 0.0032, respectively.

RESULTS

Study population. There were 8 primigravidae, 6 secundigravidae, and 9 multigravidae (2 fourth, 2 fifth, 3 sixth, 1 seventh, and 1 eighth pregnancy) in the study. Overall, 171 blood samples were analyzed, 48 from primigravidae (age range 17–23 yr), 46 from secundigravidae (age range 19–22 yr), and 77 from multigravidae (age range 21–42 yr). The mean number of blood samples analyzed per gravida was

TABLE 1

Pregnancy status and age of the study population, blood sample collection time, and number of samples analyzed

	No. women	Age range (yr)	No. samples analyzed	
			During pregnancy	Outside pregnancy
Primigravidae	8	17–23	48	8*
Secundigravidae	6	19–22	46	45†
Multigravidae	9	21–42	77	84‡
All	23	17–42	171	137§

* collected before first pregnancy.
† after first and before second pregnancy.
‡ after more than two pregnancies.
§ before and after pregnancies.

seven. A total of 137 blood samples collected before or after pregnancy were studied. Eight were from women who had never been pregnant, 45 from women who had been pregnant once, and 84 from women who had been pregnant more than once (Table 1). The mean number of samples per non-pregnant woman was six.

The age range of the 20 women analyzed during pregnancy was 17–42 years (median 21 years); 14 were $<$ 22 years old (7 primigravidae, 6 secundigravidae, and 1 multigravida) and 9 women $\geq$ 22 years old (1 primigravida and 8 multigravidae). Since age may have a profound influence on malaria and may confound the effect of parity, we analyzed in parallel the influence of pregnancy and age on various parameters. The cut-off of 22 years was chosen after multivariate analysis of the various parameters investigated here.

Prevalence and density. Thick blood film readings were done for 283 of 308 samples. *Plasmodium falciparum* was detected for 69 of 283 samples (24.3%). Table 2 shows that there were significantly more thick blood films positive for *P. falciparum* during pregnancy (29%) versus outside pregnancy (17.5%; $P < 0.03$ by chi-square test). The mean parasite density was higher during pregnancy (arithmetic mean: 98/μl) than outside pregnancy (arithmetic mean: 33/μl; $P < 0.025$ by Mann-Whitney test).

Age also had a marked influence on both prevalence and mean parasitemia in these asymptomatic women (Table 2). Thirty-five percent of the blood films were positive for *P. falciparum* in the $<$ 22 year old age group compared to 12% in the $\geq$ 22 year-old age group ($P < 0.00001$ by chi-square test). A higher parasite density was detected in the younger (arithmetic mean: 169/μl) as opposed to the older women (arithmetic mean: 9/μl; $P < 0.00001$ by Mann-Whitney test).

In blood samples taken from subjects who had experienced malaria transmission in the 60 days prior sampling (transmission group), more thick blood films were positive for *P. falciparum* and parasite densities were higher than in blood samples taken in the absence of malaria transmission in the preceding 60 days (non-transmission group) ($P < 0.02$ by chi-square test and $P < 0.02$ by Mann-Whitney test, respectively).

Combined *msp1* and *msp2* nested PCR genotyping (PCR positive for at least one locus) indicated a prevalence of 83.4% for *P. falciparum*. A discrepancy between thick blood film readings and nested PCR results has been observed in previous studies of blood samples from asymptomatic Ndiop villagers.[16,23] It is due to the large number of asymptomatic individuals harboring parasites below the microscopic detection threshold. There were more nested PCR-positive blood samples during (85%) than outside pregnancy (78%; $P < 0.024$); in the younger (90%) than older age group (75%; $P < 0.001$); and in the transmission (90%) than non-transmission group (80%; $P < 0.033$) (Table 2).

Parasite diversity. Allelic polymorphism in this population was high. All three allelic families described for *msp1* block 2 (K1, MAD20, and RO33) and both allelic families described for the central domain of *msp2* (3D7 and FC27) were detected. Overall, 17 distinct MAD20 alleles and 21 distinct K1 alleles were detected. For RO33, no size polymorphism was observed and all fragments were considered to be a single allele. For 3D7 and FC27 *msp2* allelic families, PCR fragments could be assigned to 34 and 22 distinct alleles, respectively.

The frequency of the *msp1* block 2 and *msp2* allelic families did not differ during pregnancy versus outside pregnancy or according to transmission in the previous 60 days (data not shown). The distribution of *msp2* allelic families was similar in both age groups, unlike the distribution of *msp1* block 2 allelic families which showed an association with age. The K1-type alleles were more frequent and the RO33-type less frequent in younger than older women ($P < 0.0001$ by chi-square test for both alleles). Other factors including hemoglobin type, glucose-6-phosphate dehydrogenate deficiency, and ABO and Rhesus blood group showed no influ-

TABLE 2

A comparison of results of thick blood film readings and nested polymerase chain reaction (PCR) analyses during and outside pregnancy in two age groups, and according to the presence or absence of *Plasmodium falciparum* transmission in the 60 days prior blood sampling

Method of analysis	Microscopic examination of blood slides					Nested PCR				
	No. samples analyzed	No. (%) positive	*P*—value	Mean parasite density*	*P*—value	No. samples analyzed	No. (%) positive	*P*—value	Sum of PCR fragments	Mean no. PCR fragments/sample (± SD)
During pregnancy	169	49 (29)	< 0.03	98	0.025	171	150 (85)	< 0.024	375	2.2 (1.7)
Outside pregnancy	114	20 (17.5)		33		137	107 (78)		205	1.5 (1.25)
Age $<$ 22 years†	151	53 (35.1)	$< 10^{-5}$	169	$< 10^{-5}$	173	155 (90)	< 0.001	408	2.35 (1.7)
Age $\geq$ 22 years†	132	16 (12.1)		9		135	102 (75)		172	1.27 (1)
No transmission‡	200	41 (20.5)	< 0.02	38	< 0.02	215	173 (80)	< 0.033	352	1.6 (1.3)
Transmission‡	83	28 (33.7)		194		93	84 (90)		228	2.5 (1.85)

* Parasites/μl blood (arithmetic mean, includes negative slides).
† 22 years = cut–off value.
‡ 60 days prior to blood sampling.
SD = standard deviation.

TABLE 3

Distribution of individual merozoite surface protein-1 (*msp1*) and merozoite surface protein-2 (*msp2*) alleles by age, parity, and pregnancy status

msp 1 genotyping								
			No. samples positive for each distinct allele†					
			Age		Parity		Pregnancy	
Allelic family	Fragment size (no. bp)	No. samples*	< 22 yr	≥ 22 yr	0–1	> 1	During	Outside
K1	160	12	8	4	5	7	6	6
K1	170	24	22	2	21	3	19	5
K1	180	36	30	6	26	10	27	9
K1	190	25	15	10	11	14	18	7
K1	200	25	23	2	**22**	**3**	22	3
K1	210	19	15	4	10	9	12	7
K1	220	10	6	4	5	5	5	5
K1	230	10	8	2	5	5	6	4
K1	250	12	11	1	9	3	**2**	**10**
M20	170	16	**16**	**0**	**16**	**0**	**16**	**0**
M20	190	14	12	2	11	3	12	2
M20	220	13	9	4	8	5	7	6
RO33	140	151	**88**	**63**	**76**	**75**	98	53
Rare alleles		103	81	22	61	42	66	37
Total no. of bands‡		470	344	126	286	184	316	154
3D7	210	12	5	7	5	7	6	6
3D7	230	15	12	3	12	3	7	8
3D7	240	14	12	2	10	4	5	9
3D7	250	18	6	12	6	12	6	12
3D7	260	29	17	12	14	15	12	17
3D7	270	12	9	3	8	4	6	6
3D7	280	16	12	4	12	4	9	7
3D7	290	10	10	0	9	1	8	2
3D7	300	17	11	6	10	7	17	0
3D7	330	12	10	2	7	5	7	5
3D7	350	12	9	3	10	2	8	4
3D7	370	15	11	4	11	4	13	2
FC27	380	11	5	6	3	8	0	11
FC27	420	10	8	2	8	2	9	1
FC27	480	12	10	2	9	3	6	6
FC27	490	18	17	1	17	1	15	3
Rare alleles		147	95	52	73	74	97	50
Total no. of bands‡		380	259	121	224	156	231	149

bp = base pairs.
* number of samples in which the allele was detected.
† The distribution of individual alleles detected in ≥ 10 samples is indicated. Rare alleles (observed in 1–9 samples) were grouped together under rare alleles. There were 26 rare *msp1* alleles and 40 rare *msp2* alleles.
‡ sum of PCR fragments detected in all samples for the locus considered.
Statistically significant differences in allele frequency between groups are boxed.

ence on the distribution of *msp1* block 2 and *msp2* allelic families.

Up to 18, 10, and 17 distinct *msp1* alleles and up to 17, 10, and 15 distinct *msp2* alleles were observed in primigravidae, secundigravidae, and multigravidae, respectively. The frequency of individual *msp1* block 2 and *msp2* alleles was associated with age (Fleiss and O'Quigley and Schwartz tests, $P < 0.00001$ for *msp1*; $P < 0.0001$ for *msp2*). The influence of pregnancy status on the overall *msp1* and *msp2* allele frequency was highly significant (for *msp1,* Fleiss test $P < 10^{-10}$, O'Quigley and Schwartz test $P < 10^{-9}$; for *msp2,* Fleiss test $P < 10^{-9}$, O'Quigley and Schwartz test $P < 10^{-9}$). Parity also affected *msp1* and *msp2* allele frequency (Fleiss and O'Quigley and Schwartz tests, $P < 0.0002$ for *msp1, P* $< 10^{-7}$ for *msp2*).

We restricted the analysis of the distribution of individual alleles by logistic regression to those detected ≥ 10 times. Thirteen and 16 alleles for *msp1* and *msp2* were analyzed in this manner. Data are shown in Table 3. The distribution of

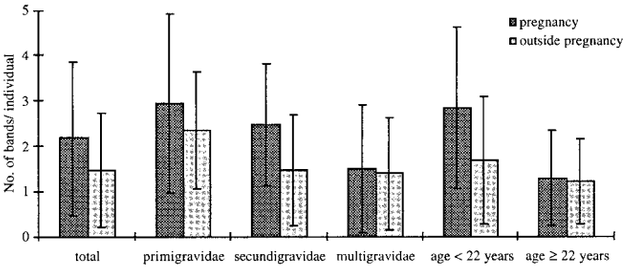

FIGURE 1. Multiplicity of infection estimated by merozoite surface protein-1 (*msp1*) block 2 and merozoite surface protein-2 (*msp2*) polymerase chain reaction (PCR) genotyping in primigravidae, secundigravidae and multigravidae, in the younger (< 22 years) and older (≥ 22 years) age groups. There were 48 and 8 blood samples in nulliparae during and outside pregnancy, respectively, 46 and 45 blood samples during and outside pregnancy in primiparae, respectively, and 77 and 84 blood samples during and outside pregnancy in multiparae, respectively. There were 97 and 76 blood samples during and outside pregnancy in the younger age group (< 22 years), and 74 and 61 blood samples in the older age group (≥ 22 years), respectively.

the 170 bp MAD20 allele, the 140 bp RO33 allele, and the 250 bp 3D7 allele differed in both age groups ($P < 0.008$, < 0.001 and < 0.002, respectively). The frequency of two *msp1* alleles was influenced by parity (the 170 bp MAD20 allele, $P < 0.003$; the 140 bp RO33 allele, $P < 0.001$). The frequency of the 200 bp K1 allele and the 490 bp FC27 allele was influenced by parity according to the exact Fischer test ($P < 0.004$ for both alleles) but not by the Wald test ($P <$ 0.01 and $P < 0.018$, respectively). The distribution of two *msp1* alleles and two *msp2* alleles differed during and outside pregnancy (the 170 bp MAD20, $P < 0.005$; the 250 bp K1 allele, $P < 0.001$; the 300 bp 3D7 allele, $P < 0.002$; the 380 bp FC27 allele, $P < 0.0001$). Thus, the distribution of the 170 bp MAD20 allele was particularly remarkable. It was detected only in younger women, only during pregnancy, and only in primigravidae.

Multiplicity of infection. Polymerase chain reaction genotyping based on *msp1* block 2 and the central *msp2* domain, which are single-copy loci, enables the detection of multiple *P. falciparum* infections. Detection of a single PCR fragment for each locus is classified as an infection with one parasite type. Detection of more than one PCR fragment for any locus reflects an infection with more than one parasite clone. Among the 257 blood samples with a positive nested PCR result, 163 (63.4%) were multiply infected. Table 2 shows that the mean number of PCR fragments per blood sample was higher during than outside pregnancy, in younger than older women (whatever their pregnancy status), and in the samples collected during or shortly after active transmission. These results indicate that both younger age and pregnancy increase multiplicity of infection.

In order to evaluate the role of pregnancy and parity on multiplicity of infection in primigravidae and/or secundigravidae compared to multigravidae, multiplicity of infection was compared for each group both during and outside pregnancy. The data are shown in Figure 1. Multiplicity of infection was analyzed using a generalized estimating-equation approach with a link function for a Poisson response, taking into account known factors influencing *P. falciparum* infections (age, parity, transmission pattern, and treatment

within the 30 days preceding blood collection). Since only one woman had the AS hemoglobin type, we did not consider this potentially confounding factor in the analysis. We also eliminated use of bed nets, blood group, G6PD deficiency, and trimester of pregnancy from the multivariate analysis since these factors did not show a significant effect on multiplicity of infection. Poisson regression analysis controlled for age, parity, antimalarial treatment, and transmission showed that blood samples collected during pregnancy had 1.43 times more PCR fragments than those collected outside pregnancy (95% confidence interval: 1.17–1.77; $P < 0.001$). The mean number of PCR fragments per blood sample was also higher in the younger than older age group (ratio 1.79, 95% confidence interval: 1.45–2.21, $P < 0.0001$). Depending on the manner used to control for age, primi- and secundigravidae status did or did not influence multiplicity of infection compared to multigravidae. Because of co-linearity between age and parity, it was not possible to separate the effect of parity from age. In other words, the increased multiplicity of infection observed in primi- and secundigravidae compared to multigravidae may be either due to an intrinsic effect of parity or reflect their younger age (and lower cumulative exposure to *P. falciparum* infections).

We next analyzed interactions between the variables considered in the multivariate analysis. A statistically significant interaction ($P < 0.036$) was observed between age and pregnancy on multiplicity of infection. For women less than 22 years old, blood samples collected during pregnancy had 1.67 times more PCR fragments than those collected outside pregnancy (95% confidence interval: 1.23–2.26; $P < 0.001$) whereas in women ≥ 22 years, blood samples collected during pregnancy had only 1.11 times more PCR fragments than those collected outside pregnancy (95% confidence interval: 0.80–1.54). This indicates that the impact of pregnancy on multiplicity of infection was mainly in paucigravidae and/or younger women. No further significant interaction was detected for the variables pregnancy, age, parity, antimalarial treatment, and transmission.

A second model that considered pregnancy status, parity, antimalarial treatment, and transmission but not age showed a significant influence of parity on multiplicity of infection (parity 0 versus parity > 1, ratio 1.65, 95% confidence interval: 1.22–2.24, $P < 0.001$; parity 1 versus parity > 1, ratio 1.31, 95% confidence interval: 0.96–1.79, $P < 0.082$). However, the effect was not significantly different during pregnancy versus outside pregnancy (term of interaction, $P = 0.267$). During and outside pregnancy, the parity 0 versus parity > 1 ratio was 1.79 (95% confidence interval: 1.31–2.45) and 1.69 (95% confidence interval: 1.16–2.47), respectively and parity 1 versus parity > 1 ratio was 1.65 (95% confidence interval: 1.13–2.39) and 1.14 (95% confidence interval: 0.75–1.72), respectively. In other words, the women with parity 0 (who will be primigravidae when pregnant) had higher multiplicity of infection than the women with parity 1 (who will be secundigravidae when pregnant) both before and during their pregnancy. The fact that the influence of parity was pregnancy-unrelated suggests that it merely reflects an age-related effect, as the women with a low parity are the younger ones.

Similar conclusions with respect to the impact of pregnancy, age, parity, antimalarial treatment, and transmission were obtained with models controlling for the effect of parasite density (data not shown).

This analysis indicated that multiplicity of infection increased during pregnancy and this was accentuated in the younger women. However, the effect of a low parity on multiplicity of infection could not be distinguished from the effect of a younger age.

DISCUSSION

Fluctuations of individual parasite types and complex dynamics of multiclonal infections have been observed during the transmission season and during the dry season in Ndiop villagers.[23] The work reported here was not intended to establish a comprehensive inventory of infecting parasite types during pregnancy but rather to compare some molecular markers of infection in women living in Ndiop during and outside pregnancy. We wanted to investigate whether the particular status of increased susceptibility to malaria in pregnant women was associated with different parasite types and multiplicity of infection. The design of the study was such that pregnant women were promptly treated when presenting symptoms of malaria and a thick blood film positive for *P. falciparum* parasites whatever the parasite density. Five antimalarial treatments were administered in the cohort studied. The five samples collected during a clinical episode were excluded from the study. We restricted the analysis to samples collected from asymptomatic women and investigated susceptibility to *P. falciparum* infection rather than clinical malaria. We studied samples collected during periods of active transmission (with frequent inoculation of novel parasite types) and samples collected during periods without evidenced transmission, where parasite carriage is chronic.

This study shows that pregnancy was associated with a higher prevalence of *P. falciparum* infection, higher parasite density, and increased multiplicity of infection. The mean number of distinct genotypes per sample during pregnancy was 2.1 compared to 1.5 outside pregnancy. This increased parasite prevalence and density reflects an increased susceptibility to asymptomatic infection during pregnancy. The calculated multiplicity of infection tended to be higher than that observed in previous work conducted in Ndiop[16,23] which is likely to be due to the increased sensitivity of the nested PCR procedure used here. The relationship between density and multiplicity can be interpreted in different ways. Felger and others proposed that the positive relationship between multiplicity of infection and density reflects a higher probability of detecting multiple clones in samples with a high parasite density.[24] An alternative interpretation is that high parasite density results from the accumulation of multiple clones. In Ndiop, parasite density and multiplicity of infection increase as transmission increases. However, when the number of parasite types per blood sample was controlled for, transmission no longer influenced parasite density (data not shown). This indicates that the primary factor influencing density in asymptomatic subjects from Ndiop is the number of clones. Since the effects of pregnancy, age, and parity on multiplicity of infection were similar regardless of parasite density, we ascribe the higher parasite density during pregnancy to an increased number of co-infecting parasite

types. This molecular marker thus indicates that pregnancy is associated with increased permissiveness to a large number of clones. This is consistent with pregnancy-associated immunosuppression. Depression of cell-mediated immunity during pregnancy[5,6] is predicted to reduce control of preerythrocytic stages, resulting in an increased number of clones completing the hepatic phase and successfully reaching the erythrocytic stage. It is also predicted to impair the response to novel, recently inoculated serotypes (e. g., *var* serotypes), resulting in increased blood-stage parasite density and greater multiplicity of infection.

This study included analysis of eight primigravidae, six secundigravidae, and nine multigravidae. It was thus possible to study women at their first pregnancy, which is associated with the greatest susceptibility to *P. falciparum.*[1,2] As primigravidae are the youngest and multigravidae are the oldest women, the analysis necessitated consideration of the potential confounding factor of age (most probably synonymous in this case with accumulated exposure). Previous cross-sectional studies of Ndiop villagers of all age groups did not identify an association of age with multiplicity of infection or allelic distribution.[16,23] However, in the cohort of women studied here, where we zoomed in on a specific age range by analyzing 308 blood samples collected from women aged 17 to 43 years, we did find a major association of age with *P. falciparum* prevalence and density and on multiplicity of infection. In Ndiop, young adults have not yet acquired anti-parasite immunity, as clinical incidence rates decrease slowly with age.[25] Thus, a decreased multiplicity of infection in the older age group, presumably reflects the higher level of acquired antiparasite immunity resulting from cumulative exposure. The highest multiplicities of infection were observed in primigravidae and in women less than 22 years old during pregnancy. Multivariate analysis indicated that both age and pregnancy status were relevant variables, but it was not possible to distinguish the effect of parity from that of age on multiplicity of infection. It should be emphasized that there was little overlap of primigravidae and multigravidae in the younger ($<$ 22 years) and older ($\geq$ 22 years) age groups. These results indicate that younger age (reduced exposure and hence limited immunity) contributes to the higher susceptibility to *P. falciparum* infection in primigravidae compared to multigravidae.[1,3] The influence of age *per se* on the increased susceptibility of primigravidae may have been overlooked in many studies.

The genetic diversity of *P. falciparum* parasites analyzed here, collected over six years, was large. The distribution of individual *msp1* and *msp2* alleles was influenced by pregnancy. Importantly, a highly skewed distribution of specific individual alleles, such as the *msp1* Mad20–170 bp or the *msp2* FC27 490 bp allele was observed in primigravidae. This is consistent with infection by specific *P. falciparum* subpopulations with placental tropism during first pregnancy and subsequent elimination of those parasites by specific immune responses.[7,8] A mean number of eight blood samples per gravida were analyzed. Parasite diversity during pregnancy was high, with up to 18 distinct *msp1* alleles and 17 distinct *msp2* alleles observed throughout pregnancy in one case. Thus, during the course of pregnancy women experience infection by a large number of distinct strains. This allelic diversity is yet another reflection of the increased susceptibility of pregnant women to malaria.

In conclusion, our results indicate that pregnancy is associated with an increased prevalence of asymptomatic *P. falciparum* infections, an increased parasite density, and infection by numerous parasite types, including infection by specific types in primigravidae. These observations are consistent with a reduced capacity to control parasite numbers due to immunosuppression and preferential infection by specific types in primigravidae.

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