CHAPTER 3
Prevalence of malaria and risk factors associated with malaria in semiurban district of Hazaribag, Jharkhand, India.

Introduction

Tropical malaria, which is caused by the protozoan parasites *Plasmodium falciparum* and *Plasmodium vivax*, accounts for 515 million clinical cases [214] and 1 to 3 million deaths annually [215]. *Plasmodium vivax*, the most widespread parasite causing human malaria, is responsible for an estimated 130–435 million infections annually and is the major cause of malaria in most of Asia and Latin America [216]. Although *P. vivax* infection is commonly regarded to be much more benign than *Plasmodium falciparum* infection, historical evidence advocates significant mortality associated with *P. vivax* malaria in the pre-antimalarial era [217], and death caused by *P. vivax* malaria has been alarmingly recognized over the past few years [216,218].

The emergence and spread of drug resistance to commonly used chemotherapeutics are major factors contributing to this increasing burden and most of the mortality and morbidity is borne by children and pregnant
women. Pregnant women and their infants are susceptible to common and preventable infectious diseases including malaria but are woefully left unscreened and untreated. According to an estimate, approximately 125 million pregnant women worldwide are exposed to the risks of malaria in pregnancy (MIP) each year, resulting in 200,000 infant deaths [219]. Every year, in India, 28 million pregnancies take place with 67,000 maternal deaths (Registrar General of India. Sample Registration System. Special Bulletin on Maternal Mortality in India. 2004-06), 1 million women left with chronic ill health, and 1 million neonatal deaths [220]. Pregnancy is an event of immunologic tolerance, whereby a woman accepts the implantation of the fetal allograft in her uterus and a unique period for vulnerability to malaria infection. Pregnant women with relatively low levels of previously acquired immunity are particularly at high risk of the most severe complications of malaria during pregnancy, such as cerebral malaria, severe malaria anaemia, abortions, intrauterine fetal death, premature delivery, stillbirths, and maternal and infant mortality [219, 221]. In malaria-endemic areas, pregnant women are more susceptible to Plasmodium infections than their non-pregnant peers. The adverse outcomes of these infections are primarily felt by primigravidae [222,223], although in areas of low or unstable transmission, women of all gravidities may be equally at risk [223]. Pregnant women are 3 times more likely to suffer from severe disease as a result of malarial infection compared with their non-pregnant counterparts, and have a mortality rate from severe disease that approaches 50% [224,225].

In spite of severe and fatal consequences of malaria during pregnancy for the mother, foetus, and newborn child, the harmful effects can be substantially preventable and reduced [226] by using either available interventions or through appropriate treatment upon early and stringent diagnosis [227, 228]. Because malaria infection during pregnancy is often asymptomatic, the most common control strategy is intermittent preventive
treatment during pregnancy (IPTp) - designed to clear any malaria infection present at the time of treatment and also to provide post-treatment prophylaxis to prevent infection for a period of weeks. However, increasing concern of widespread resistance of commonly used antimalarial drugs[229] over the globe has opened the avenues for alternative and effective interventions. The diagnosis of malaria during pregnancy is complicated by several factors, including multi stage pregnancy terms lacerated with diminished immunity, increased susceptibility of sever diseases, various obstetric complications, splenic and placental sequestration of parasites, various forms of anaemia and variation in patient presentation. Thus, development of prompt and accurate diagnosis is an important goal of MIP research.

*P. falciparum* malaria during pregnancy is a well-known cause of maternal and fetal morbidity and mortality. Although *P. vivax* infection has received less attention than *P. falciparum* infection, it is clearly an important contributor to both maternal anaemia and low birth weights [230-232] were they frequently coexists. However, of 50 million pregnancies occurring each year in countries where malaria is endemic, approximately one-half occur in areas where *P. vivax* malaria is endemic [226]. Although *P. vivax* infection during pregnancy has been recognized for many years [230], the impact of such infection during pregnancy has been assessed only recently. In series from Thailand and India, women with *P. vivax* infection were more commonly anaemic and delivered lower birth weight neonates, compared with uninfected women, but the effects were less pronounced than those associated with *P. falciparum* infection [231, 232]. In both studies, *P. vivax* infection was most common during the first pregnancy, and the prevalence of such infection peaked early during the second trimester.

Limited and past MIP studies in India have demonstrated the important contribution of malaria to maternal and neonatal morbidity and mortality.
Although preliminary results from earlier studies carried out primarily in central India suggest that both *P. falciparum* and *P. vivax* are associated with adverse pregnancy outcomes, these studies primarily focused on symptomatic pregnant women infected with vivax [231,235]. Relatively little information is available from India about vivax associated malaria during pregnancy, particularly from Jharkhand, an understudied and tribal dominant region with perennial malaria transmission zone where malaria is rampant and causing sizable annual malaria deaths, second to Orissa in India as per the latest observation published by Dhintra et al. [236] and Hussain et al. [237], which reflects the importance of the area and its necessity of undertaking extensive investigation in terms of malarial pathology, is concerned and by Hamer et al. [233] reflecting the malaria during pregnancy associated with an increased risk of neonatal and infant mortality.

Thus, in view of the limited information on asymptomatic and vivax infection during pregnancy in India, prompted us to investigate with an objective to better define the estimate of MIP, the prevalence of asymptomatic malaria, and the relative contribution of *P. falciparum* and *P. vivax* during pregnancy and at delivery. To the best of our knowledge, such profile, epidemiological association and clinical correlation has not been investigated before on isolates of malaria in pregnancy from Hazaribag, Jharkhand, among malaria endemic regions of India. Most significantly, our investigation will be the first report attempting to evaluate the interplay among anaemia, pregnancy and asymptomatic malaria, stratified according to clinical groups in adult population residing in a perennial transmission zone with a co-dominance of *P.vivax* and *P. falciparum* prevalent region. The study was conducted at Hazaribag in the state of Jharkhand in east India, with the ultimate goal of enhancing the development of evidence-based policies to reduce the burden of disease due to MIP in this region of India.
Methods

Study Sites/design and Population

The study consisted of cross-sectional surveys conducted in three units, i.e. antenatal care units (ANC), delivery units (DU), or the inpatient antepartum ward of Sadar hospital in Hazaribag districts of Jharkhand, India. Jharkhand had a yearly average slide positivity rate (SPR) for symptomatic individuals of 6.8% over the last three years with *P. falciparum* accounting for 44% of the cases [238]. The province of Jharkhand in eastern India is one such area where malaria is rampant. The complexity and magnitude of malaria in the central eastern part of India deserves special mention and attention as the central eastern state contributes 15–20% of total malaria cases in the country as per the Draft on National Policy on Tribals by Govt of India, 2005. The investigation is conducted in the Jharkhand state emphasizing tribal dominant area (total population according to 2001 census is 31 463 866), and the state of Jharkhand is selected to represent an endemic with stable transmission of malaria, with a total of 230 686 malaria cases reported in 2009, of which 39.53% were due to *P. falciparum* [239]. The present study was carried out at Hazaribag district, considered to be a malaria-endemic area in the state of Jharkhand.

Hazaribag (total population according to 2011 census is 1,734,005) is selected to represent a rural-cum-semi urban district with low but perennial transmission of malaria. Hazaribag had a yearly average SPR of 7.3% for symptomatic individuals over the last three years, with *P. falciparum* accounting for 14% of the cases [240]. The majority of the indigenous population is mix of tribals, schedule caste, schedule tribes and other casts; exceptionally typical social stratification having gender disparity.
Moreover, the district and state lies in the tropical zone with an annual rainfall of 1234.5 mm with favorable geo-climatic and ecological conditions conducive for perennial malaria transmission. The climatic conduciveness of the investigated district can be best visualized in the self explanatory Supplementary Figure-1A. Most interestingly, the monthly climatic temperature when compared with monthly malaria episode, we observed significant correlation between ambient temperature and subsequent rise and fall in malaria episode as shown in Supplementary Figure-1B. The recent (2010-2012) data on malaria epidemiology has been analyzed during investigation in this project and we observed the increasing trend of malaria episodes as shown in Supplementary Figure-2A-C; despite of consistent interventions and preventive measures had been implemented by various national and international bodies. Thus, the selected study district is the true representation of typical conditions that would be found in malaria endemic districts of Jharkhand. The District Level Household and Facility Survey conducted between December 2007 and April 2008 revealed that 56% of women had at least one antenatal clinic (ANC) visit and 18% overall had institutional deliveries including 59% in urban areas but only 13% in rural settings[241]. Sadar Hospital, the district hospital for Hazaribag District, serves a predominantly rural population and has a separate obstetric unit with 40 beds, with a high volume of annual deliveries ranging from an average of 4800 to 5500 per year in 2010 to 2013. The Sadar Hospital also has a high volume of ANC visits including an average of 5200 to 6600 per year from 2010 to 2013.

**Screening and enrolment**

The study had three components recruiting distinct group from ANC, delivery units (DU), or the inpatient antepartum ward. For the ANC
component, pregnant women aged ≥17 years who reported to the study site for routine care were screened and enrolled, those were willing and consented to participate in our study. For the DU component, women aged ≥18 years who presented for delivery and were willing to provide written informed consent were enrolled. For inpatients, pregnant women with an admission diagnosis of malaria, anaemia, or a febrile illness of unknown origin were screened for study participation. Those whose malaria-related diagnosis was confirmed were enrolled after obtaining informed consent. All the women at each attendance underwent clinical investigations, parasite slide examination and measurement of auxiliary body temperature before enrollment and detailed strategy of enrollment; sampling procedures and broad groups were classified as shown in schematic flow chart in Figure-2.

**ANC procedures**
Trained study personnel interviewed the enrolled women and collected information on socio-demographic characteristics (i.e., date of birth, socio-economic status, literacy); reproductive history including gravidity; history of fever and anti-malarial drug use; and use of anti-malarial prevention measures. A complete physical examination including the determination of gestational age was assessed by palpation of uterine fundus height combined with information on last menstrual period; measurement of auxiliary temperature with digital thermometer, and other vital signs was also performed. Peripheral venous blood (3–5 ml) was collected from all the attendee for malaria blood film preparation, rapid diagnostic test (RDT) and haemoglobin determination apart from other biochemical and molecular investigations. Women with positive RDT results or who were anaemic were referred immediately to the hospital physician for treatment. The hospital staffs were informed of additional parasitaemic individuals identified through blood smears so that they could be appropriately treated.
DU procedures

Pregnant women enrolled at the DUs were interviewed, with data collection focused on socio-demographic and anthropometric characteristics, obstetric complications, history of fever and anti-malarial use during pregnancy, and the use of anti-malarial prevention measures, birth outcome and mode of delivery. Peripheral venous blood (3–5 ml) was collected after delivery for malaria blood film preparation, and or rapid diagnostic test (RDT), and haemoglobin determination apart from other biochemical and molecular investigations. Women with positive RDT or blood smear results were referred for treatment. Apart from malaria prevalence study in DU, we have also collected clinical and demographic data and samples based on the mode of delivery i.e. normal, caesarean and still birth delivery and further on the mode of birth/delivery outcome i.e. pre-term, post-term and term delivery; details were presented in Table-1.

Inpatient procedures

Enrolled subjects were interviewed and information on socio-economic status, reproductive history including obstetric history, history of fever and anti-malarial drug use, and the use of anti-malarial prevention measures, birth outcome and mode of delivery were collected. Data including recorded clinical signs, results of laboratory investigations, treatments administered, admission and discharge diagnosis and outcome of admission were procured from the subject's hospital record.

Laboratory procedures

Thick and thin smears prepared from peripheral blood of ANC and DU subjects were Giemsa-stained and examined under high power. The parasite density was evaluated by counting the number of asexual forms of parasites for every 200 leukocytes, assuming a leukocytes count of 8000
leukocytes/μl of blood[242]. The thin film was used to identify the Plasmodium species. All slides were cross-checked using stringent diagnostic criteria to diagnose plasmodium infection with our trained technical staff. The commercial (RTD kit) First Response Malaria pLDH/HR2 combo test kits (Premier Medical Corporation, Mumbai, India) were also used as per the manufacturer's guideline as a screening tool for diagnosing malaria in pregnant women.

**Haemoglobin Concentration**

Haemoglobin (Hb) levels were recorded at the first ANC and DU visit. Determining the concentrations of haemoglobin (Hb) were performed in peripheral blood samples using a portable HemoCue haemoglobinometer (HemoCue AB, Ängelholm, Sweden) as stated by the manufacturer. The concentration of Hb was recorded on the study questionnaire and double-checked by the laboratory technician. Women were classified as anaemic (Hb<11g/dl.) and then categorized as being moderately to severely anaemic haemoglobin <8 g/dl and<7g/dl, respectively as the primary outcome, and being mildly to non-anaemic (Hb≥9g/dl.) according to WHO classification of anaemia [243].

**Study definitions**

Malaria was defined as the presence in the peripheral blood of asexual blood stage of Plasmodium or positive RDT, irrespective of species or symptoms. Symptomatic malaria infection was defined; when history of fever within the last week or temperature ≥ 37.5°C associated with the presence of asexual forms of *P. falciparum* or *P. vivax* on thick blood smear or a positive RDT. Severe malaria was defined as a malaria attack associated with any of the following; cerebral malaria, severe anaemia, renal failure, pulmonary oedema, hypoglycaemia, shock, spontaneous bleeding, or repeated convulsions [244]. Maternal height and weight were
taken at the first visit to ANC and DU, based on this information the body mass index (BMI) were calculated as weight (kg) divided by the squared height (meters); a low BMI was defined as a BMI < 22.0 kg/m². A documented fever was defined as an auxillary temperature ≥37.5 °C.

Ethics Statement and Subject Consent
All human blood samples used in this study were collected after obtaining written consent from the study participants under protocols activities approved by the Institutional Ethics Committee (IEC) of the Vinoba Bhave University, Hazaribag, Jharkhand and human ethical guidelines as reflected in the guidelines of the Medical Ethics Committee, Ministry of Health, Govt. of India. All study participants provided informed consent. The protocol is approved from IEC, VBU having memo no. VBU/R/888/2012 dated 05-06-2012.

Data management and analysis
All clinical, demographic and anthropometric information were carefully checked for correctness and inconsistencies were resolve before analysis. Data were entered in MS-Excel and analyses were performed using SPSS v.16 (SPSS Inc., Chicago, IL, USA) and Graphpad Prism version 5.0 (GraphPad Software, Inc., CA, USA). For comparisons of means between two groups of subjects, the student’s t-test were used for normally distributed data and when data were not normally distributed; non-parametric tests (Mann-Whitney U) were used to analyze the data. Categorical data are presented as frequency counts (percent) and compared using the chi-square or Fisher's exact statistic as appropriate. Continuous data are presented as means (± standard error) and compared using the t-test or analysis of variance as appropriate. Since most participants did not know their exact date of birth, we have presented participants' ages in ranges based on their estimations. Risk factors for either *P. falciparum* or
*P. vivax* parasitemia were evaluated by univariate analysis and then adjusted for significant predictors in multivariate analysis. The differences were considered statistically significant when the *P* value obtained was <0.05.

**Results**

Recruitment and enrolment took place from September 2012 to December 2013. Of 1890 pregnant women screened during their ANC visits, 1746 were willing to understand our study protocol, out of which 1715 were consented and agreed for peripheral sampling and 31 refused to participate in the study. Thus, we enrolled 1715 subjects and upon pregnancy screening report, interviewed by trained technical staff and based on other clinical investigations; divided them into the two broad groups, i.e. pregnant and non-pregnant women group consisting of 1271 and 444 subjects, respectively. Further, pregnant group were sub-divided on the basis of first, second and third trimester that consisted of 135, 492 and 644 subjects, respectively. The non-pregnant group was sub-divided into women with malaria and healthy women without malarial complications, consisting of 227 and 217, respectively. In the delivery unit, 870 pregnant women were screened and were enrolled.

**Antenatal clinics**

Most pregnant women attending ANC were in the 18 to 38 year old age range and had some level of formal education (Table 1). The vast majority of participants were Hindi speaking (97.6%) and non-smoking (98.7%). Most owned their own home (75.4%) and were engaged in household work (76.7%) with a small proportion involved in farming (12.3%). They had attended a median of one ANC visit (range 0-9) during their current pregnancy and almost one third of the attendee was primigravidae (33.3%). Slightly more than half of participants presented to the ANC in the latter
half of pregnancy whereas 44.6% presented prior to 20 weeks. Less than half of the participants reported taking iron/folate supplements (46.3%) while 33.2% were taking multivitamins. In terms of malaria prevention activities, most pregnant women reported having untreated bed nets in their homes, and using them recently, but very few had ITNs (Table 2). Similarly, only 9 of the women were taking prophylaxis for malaria and most of them (7/9, 78%) were unable to identify the drug they were taking and rest (2), who were able to identify the drug, were taking chloroquine. A positive diagnostic test for malaria was obtained in 5.4% (68/1271) of the total cohort (Table-3). Blood smears for malaria were positive in 4.3% of pregnant women while an additional 14 (1.1%) women had positive RDTs. The mean density of parasitemia in the 54 women with positive blood smears was 63,236 asexual forms/µl (range 600-489,000). *P. falciparum* was identified in 4.4% of parasitaemic individuals while *P. vivax* was found in 86.8% and 8.8% of infections were mixed. Peripheral parasitemia was over four times more likely among women living in rural areas when compared with those from urban or semi-urban subjects (OR 4.32, 95% CI 1.67-9.46), and among primigravidae and secundigravidae relative to multigravidae (OR 4.75, 95% CI 1.23-11.58). Parasitaemia was more commonly encountered in pregnant women who had a history of fever within the week prior to enrollment or were febrile at the time of the study visit (4.2% vs. 2.3%, p=0.02). Most interestingly, 70.6% (48/68) of the pregnant women with a positive malaria diagnostic test at the time of ANC visit were asymptomatic. The majority of positive malaria tests occurred from July to January with the greatest number in between August to October, corresponding to the monsoon season. Further multivariate analysis was performed in order to identify the association between specific demographic, socioeconomic, and malaria prevention activities and the risk of parasitemia. Among pregnant women attending ANCs, first/second pregnancies, fever in the past week, and residences in rural
areas were significantly associated with peripheral parasitemia as shown in Table-4.

**Delivery units**

Like the ANC cohort, most pregnant women attending DUs were aged 20-36 years and had some level of formal education (Table-1). All were non-smokers (100%) and nearly all spoke Hindi (97.2%). Most owned their own home (73.9%) and were involved in household work (84.3%); a minority engaged in farming (14.6%). Study participants had attended a median of three ANC visits (range 0-9) and about slightly less than two-thirds were primigravidae and secundigravidae (Table-1). The majority of pregnant women reported having untreated bed nets in their homes and using them recently but ITN ownership was uncommon (Table-2). Only three women were taking chemoprophylaxis for malaria and none knew the name of the medication that they were taking.

Only 4.3% of the women enrolled at the DUs had peripheral parasitemia (either a positive blood smear and/or RDT). *P. falciparum* was identified in 5.4% (2/37), *P. vivax* in 86.5% (32/37), and mixed infection in 8.1% (3/37). The mean density of parasitemia in the women with positive blood smears was 16,395 asexual forms/μl (range 870-65,000). The peripheral parasitemia density was significantly higher for primigravid women than in those who had one or more prior pregnancies (mean ± SD of 36,600 ± 9,743 vs. 7,532 ± 4623 asexual forms/μl, respectively; p=0.002). Asymptomatic malaria infections were present in 75.7% of women with peripheral parasitemia (28/37) and 24.3% of those with symptomatic infection (9/37). Pregnant women with peripheral parasitemia were more likely to have either a self-reported fever or fever measured at enrollment than those who were aparasitaemic (36.4% vs 9.2%, p=0.005). A sizable proportion of women presenting to the rural origin were parasitaemic as compared to semi-urban and urban origin and this difference was
significant (OR 3.46, 95%CI 1.29-10.4, p=0.03) (Table 3). Primigravidae and secundigravidae also were more likely to be parasitaemic, and difference was significant (OR 4.23, 95% CI 1.97-23.2, p=0.04). Asymptomatic malaria infections were present in 70% of women with peripheral parasitemia (26/37) as compared to 30% symptomatic infection (11/37). Pregnant women with peripheral parasitemia were more likely to have either a self-reported fever or fever measured at enrollment than those who were aparasitaemic (28.3% vs 9.2%, p = 0.004).

As observed in the ANC participants, most episodes of parasitemia occurred in July to September during the monsoon season. For DU participants with peripheral parasitemia, 83.7% had anaemia as compared to 47.6% of those who did not have parasitemia (p=0.004). More women with peripheral parasitemia had severe anaemia (5.7%) than those without parasitemia (2.6%) and the difference was significant (p=0.02).

Multivariate analysis revealed a significant association between peripheral parasitemia and primigravidae and secundigravidae, fever within the last week and semi-urban and rural residency status as shown in Table-5.

**Association between pregnancy and asymptomatic P. vivax with haemoglobin**

Anemia is the most prominent hematological manifestation of malaria infection. Hemoglobin concentration is the best characterized method and well accepted indicator for diagnosis of anemia and assessment of severity. In addition to this, it is regarded as one of the most serious global public health problems which prompted us to investigate the status of hemoglobin and severity of anemia in Jharkhand population; as anaemia is particularly high for women with no education (74%), women from the scheduled tribes (85%), and women in the two lowest wealth quintiles (over 70%). The prevalence of anaemia among adults in Jharkhand is higher than in almost all other states in India (National family health survey, NFHS-3.
Anaemia was prevalent among ANC participants whereas severe anaemia was reasonably observed in the investigated cases (Table-3). Anaemia was significantly associated with malaria (p=0.02); however, severe anaemia was more common among women with parasitemia (p=0.001). More than two third of the DU participants were anaemic whereas 16.3% had severe anaemia (Table-3). Of these ANC and DU participants, the prevalence of mild, moderate and severe anaemia are shown in Figure-3. Further, mean haemoglobin concentration was 9.7± 1.3 g/dl and the prevalence of anaemia in ANC was 84% (95% CI: 43.53-97) as compared to 83% (95% CI: 41.27-84.18) in DU. In multivariate analyses, asymptomatic malaria increased over five times the likelihood of having anaemia (aOR 5.64 95% CI: 1.56-14.75; p=0.002).

**In-patients**

Forty-five pregnant women who were admitted and treated for malaria according to national guidelines in Sadar hospital, Hazaribag, were enrolled. They represented 22.5% of pregnant women admitted for any medical treatment other than deliveries during the study period. The majority (78%) of the inpatients were between 22 and 39 years old. Thirteen (28%) were primigravidae, 18 (40%) secundigravidae, and 14 (31%) multigravidae. The sizable population was from scheduled tribes (42%) with the others belonging to general caste (35%), scheduled caste (10%) and other backward castes (13%). Similar to the ANC and DU participants, 68% reported having a bed net in the household and 46% reported sleeping under it most nights although none were having insecticide-treated bed net. Twenty-three percent of their homes had been sprayed with insecticide and none of the woman was reported for taking chemoprophylaxis during pregnancy.

Malaria was confirmed by microscopy or RDT in 84.4% (38/45) of the inpatients. *P. vivax* was the cause of malaria for 76% (29/38) of the
pregnant women, while six (16%) had *P. falciparum* and three (8%) had mixed infection with *P. falciparum* and *P. vivax*. Five (13.1%) of the admitted pregnant women had severe malaria (severe anaemia, cerebral malaria) based on WHO criteria [245]. Of the 38 patients whose haemoglobin results were recorded in the medical records, 31 had anaemia, six had severe anaemia, and only two were not anaemic. *P. vivax* was responsible for severe malaria in four of five women, while the last had a mixed infection. All admitted pregnant women were treated with parenteral or oral quinine, arteether or artesunate with excellent responses.

**Discussion**

The estimate of malaria in pregnancy continues to be grave concern for community reproductive health care management across the globe including India, upto the level of pacifying the concept of healthy mother and healthy baby of National Family Welfare Programme. In fact, situation is much more aggravated in developing countries like India, where poverty, illiteracy, geographical diversity, socio-economic disparities and multiple pregnancies take their toll of mother’s health.

Among the prominent findings of the present study, we found 5.4% and 4.3% malaria during pregnancy at ANC and DU unit, respectively as compared to only 1.8% and 1.7% at ANC and DU unit, respectively reported by Hamer et al. [233] from the series of cross-sectional and multicentric study in Jharkhand. However, our study design is slightly broader than the earlier investigation from Hamer et al. [233] in terms for subject stratification, as we also taken into the account of women with malaria without pregnancy and the prevalence of malaria were found to be 13.2%, which itself reflects the importance of the investigated region and population under malaria sensitive zone. However, our study lacks the difference of investigating placental malaria. The pondering difference in the prevalence of malaria during pregnancy between our investigations,
though we have selected only one centre in one district, i.e. Hazaribag, Jharkhand as compared to three centres from two districts i.e. Ranchi and Gumla of Jharkhand by Hamer et al.[233], may be attributed to various other reasons but primarily linked to the selection of study sites. As Ranchi, is an urbanized capital with lots of high-tech development in and around the city, local and buffering populations are much more educated, aware of practicing healthy life style and various diseases prevention strategies including malaria, high socio-economic status, excellent with a choice of health facility compared to the rest of the districts of Jharkhand state and most importantly less malarious than almost 20 other districts of Jharkhand as far as malarial epidemiology is concerned in last ten years [239]. Thus selected site by Hamer et al. [233] may not be the true representation of the malaria scenario rather burden of malaria during pregnancy in Jharkhand but absolutely true as far as the outcome of the project is concerned. Though, our results of higher prevalence of malaria in pregnancy are in accordance with the earlier observations (ranging from 1.7% to 20%) across the India [231, 233, 246, 247]. Most of these studies focused on pregnant women with selective approach, tends towards screening for mostly febrile or had a recent history of fever cases and thus may have had a selection bias towards expecting higher malaria rates. This approach, targeting malaria diagnostic and treatment for symptomatic pregnant women, is consistent with India's National Vector Borne Disease Control Programme guidelines [248]. In contrast, all pregnant women were evaluated in the current study regardless of classical symptoms and interestingly, we observed well over 70% of the pregnant women in ANC and DU had asymptomatic malaria during pregnancy, which suggests the region specific intervention. The broader spectrum of screening strategies were in accordance with earlier investigation in this region [233], though our observations are notably varied from their observations as far as asymptomatic malaria during pregnancy is concerned.
The higher prevalence of malaria in women without pregnancy and with pregnancy, irrespective of ANC and DU attendee’s location of residence i.e. rural, urban and semi-urban, suggests that Hazaribag and its buffering zone have perennial rate of malaria transmission. Therefore, population of all age groups including pregnant women are at potential risk of getting malaria infection even irrespective of transmission season, though peak was observed in post monsoon season. Apart from this, there is significant lack of education, general awareness towards health issues, congenial environmental factors for vector growth and survival and most importantly sizable population lack access to vector control methods or limited access to antimalarial drugs. People residing below poverty line linking to malnutrition and anaemia may be plausible reasons for various opportunistic infectious diseases including malaria.

Interestingly, Insecticide residual spray (IRS) of home, which is usually conducted by government agencies, was reported more in rural areas as compared to urban and semi urban zone of Hazaribag, though its seasonal usage of IRS in those areas regarded as perennial transmission may suggestive of vector resistance and subsequent higher prevalence of disease. Our observations warrant the potential need to enhance the IRS and distribution of ITNs in and around the investigated district. Overall, there was significant burden of anaemia among women in Jharkhand and particularly during pregnancy [233]. Our observations regarding anaemia are in accordance with the findings from other study in Jharkhand [233], across India [249,250] and most relevant study by Nosten F et al.[251] in which they have demonstrated that women who had malaria at any time were more likely to be anaemic than women without malaria. Thus, regardless of transmission level and the level of pre-pregnancy immunity against malaria, maternal anaemia remains the most frequent adverse consequences of malaria during pregnancy[252]. The symptoms and complications of malaria in pregnancy vary according to malaria
transmission intensity in the given geographical area, and the individual’s level of acquired immunity. In low-transmission settings, where women of reproductive age have relatively little acquired immunity to malaria, MIP is associated with anaemia, an increased risk of severe malaria. This may lead to spontaneous abortion, stillbirth, prematurity and low birth weight [253, 254]. In such settings, malaria affects all pregnant women, regardless of the number of times they have been pregnant. In pregnant women, additional sequestration of malaria infected erythrocytes occurs in the placenta. Pregnant women therefore suffer disproportionately from severe anaemia as a result of infection [226]. Our observation is also substantiated by the fact that the majority of malaria infections in pregnancy remains asymptomatic or pauci-symptomatic, yet are a major cause of severe maternal anaemia and low birth weight, especially in the first and second pregnancies [232, 233]. In areas with stable, but low transmission like ours investigated area, and certainly in areas with unstable and exceptionally low transmission, infections can become severe in all gravidae groups because most women of childbearing age in these regions have low levels of pre-pregnancy and pregnancy-specific protective immunity to malaria[226].

High prevalence of anaemia was observed and strongly correlated with asymptomatic \textit{P. vivax} infection. This prevalence is similar to those reported by Brutus \textit{et al.}[255] and Douglas \textit{et al.}[256]. Recent work has shown that in Papua New Guinea and Papua, Indonesia, mixed infection causes more severe haematological impairment than infection with either species alone [257,258]. The impact of \textit{Plasmodium vivax} infection on haemoglobin concentration varies from negligible to dramatic [257, 259-261]. The clinical consequences of the reduction in haemoglobin depend on the haemoglobin concentration prior to infection. Although the spectrum of anaemia seen with vivax infection is reasonably well documented, the clinical, developmental, and socioeconomic
consequences are largely unknown. Population-based estimates of mortality in severely anaemic individuals with vivax malaria have not been established but recent studies from Latin America, New Guinea and the Indian subcontinent have identified deaths in patients with severe vivax anaemia [257, 259, 262]. Though authors did not establish the extent to which anaemia contributed to those deaths.

The very low rate of ownership of insecticide treated bed nets (ITNs) and awareness suggests that this component of the enhanced malaria control programme (EMCP) has not effectively reached this vulnerable population although it was encouraging to find that many households had bed nets and that they were used on a regular basis. However, our investigation suggests that approaches for ITN distribution and enhancing community awareness about the importance of their use, need to be addressed as similarly observed and proposed by earlier investigation in adjacent region [233].

Despite the change in drug policy in 2008 in the studied state (Jharkhand) the availability and implementation of combination therapy i.e. artesunate plus sulfadoxine pyrimethamine is a major concern. It has been well documented that Chloroquine resistance has been rising in India [263-266]; this drug was recommended for malaria prophylaxis in pregnant women in high risk areas as reported by Hamer et al.[233], though it has been discontinued since recommendation. Presently, quinine sulphate was recommended for malaria prophylaxis in pregnant women in the investigated area irrespective of gestational age. Though this is partly in accordance with The Directorate of National Vector Borne Disease Control Programme (NVBDCP) and current WHO guidelines suggesting prophylaxis for trimester based treatment of malaria during pregnancy as quinine for first trimester and subsequently ACTs in the second and third trimester of pregnancy (http://www.nvbdcp.gov.in/Doc/Diagnosis-Treatment-Malaria-2013.pdf). Since the intensity of transmission and the prevalence of malaria in pregnant women in Jharkhand are comparatively
lesser than in many areas in sub-Saharan Africa. Notably, sulphadoxine-pyrimethamine was commonly used in Africa as intermittent preventive treatment of pregnant women (IPTp) [227], which may not be presently suggestive priority for Jharkhand to implement IPTp though may be considered as an alternative to the priority failure strategy. The top priority for Jharkhand should be on preventive measures like improved availability, awareness and uses of ITNs by pregnant women and well organised IRS system. In addition, we recommend much more stringent and frequent screening and diagnosis using conventional and RDTs irrespective of classical malaria symptoms to pregnant women in all the trimesters. Most importantly, in view of sizable prevalence based on hospital study and potential risk for population at large in the investigated region, we are also suggestive of dedicated active and passive surveillance for MIP at the community level like regular malaria surveillance under India’s NVBDCP. This strategy alone could potentially reduce the burden of MIP while limiting the potential for anti-malarial resistance to develop due to the widespread use of drugs for chemoprophylaxis. The present study shows two important findings; that, the temporal and spatial distribution of asymptomatic infections differ from that of symptomatic disease and that *P. vivax* infection and pregnancy synergistically contribute to maternal anaemia in a low and perennial malaria transmission setting.

One major limitation of this study is that we could not be able to access the placental malaria due to limitation of our study design. Although the study was restricted to women delivering in the hospital, sizable number of (more than 60%) women give birth outside Sadar Hospital, Hazaribag.

Further, a longitudinal study instead of cross-sectional would have provided better estimate of MIP in this region and probably our study design may have given underestimate as compared to actual risk population. This has also been apprehended and suggested by Hamer et al.[233]. Despite these limitations, this study provides important data on
the epidemiology and clinical implications of vivax malaria during pregnancy and delivering at Hazaribag district Sadar hospital. In spite of restricted and facility based study, we preferentially covered marginalized, tribes and remote population of the investigated semi-urban cum rural district, Hazaribag. The majority of the districts and particularly malaria endemic districts in Jharkhand have similar geographical, socio-economic, demographic, literacy, basic amenities including health facility and awareness. Thus, our observation may be utilized for baseline information for further comprehensive and multi-centric study design; in strengthening MIP associated preventive measures and screening methods within the state of Jharkhand.

Conclusion

As the global control and elimination of malaria progresses, *P. vivax* is set to become the dominant *Plasmodium* species[267], yet the health, developmental and socioeconomic consequences of vivax malaria and vivax–associated anaemia have received very little attention. This study reports a high prevalence of anaemia among pregnant women in the Hazaribag, Jharkhand and anaemia is strongly correlated to asymptomatic *P. vivax* infection. The results are quite indicative and emphasize the need to actively diagnose and treat malaria infection during ANC visit in the areas of perennial transmission. In view of the population at risk in this malaria-endemic region of India, there is a need to enhance ITN use and awareness for the prevention of MIP and distribution of ITNs at first ANC visit will be lucrative alternative. There should be a focus on improving case management of asymptomatic pregnant women, and evaluating the efficacy and effectiveness of the intermittent screening and treatment strategy. Further research is urgently needed to understand the nature and
distribution of asymptomatic malaria infection serving as an important infected reservoir to continue malaria transmission. Our finding highlights the public health importance of integrated genus-wide malaria control strategies using diagnostic tests including RDTs and ensuring the availability of safe and effective drugs for the treatment of pregnant women in areas of *Plasmodium* co-endemicity.
Table-1  Baseline characteristics of pregnant women attending antenatal and delivery units

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Antenatal clinics n=1271</th>
<th>Delivery units n=870</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>N, (%)</td>
<td>N, (%)</td>
</tr>
<tr>
<td>Age (Years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>166(13.1)</td>
<td>109(12.5)</td>
</tr>
<tr>
<td>20-34</td>
<td>983(77.4)</td>
<td>708(81.4)</td>
</tr>
<tr>
<td>≥35</td>
<td>122(9.5)</td>
<td>53(6.1)</td>
</tr>
<tr>
<td>Prior pregnancies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primigravid</td>
<td>423(33.3)</td>
<td>338(38.38)</td>
</tr>
<tr>
<td>Secundigravid</td>
<td>578(45.5)</td>
<td>209(24.1)</td>
</tr>
<tr>
<td>Multigravid*</td>
<td>270(21.2)</td>
<td>323(37.1)</td>
</tr>
<tr>
<td>Gestational age at enrollment (weeks)**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20 weeks</td>
<td>567(44.6)</td>
<td>n/a</td>
</tr>
<tr>
<td>20-36 weeks</td>
<td>641(50.4)</td>
<td>57(6.5)</td>
</tr>
<tr>
<td>≥37 weeks</td>
<td>63(5)</td>
<td>813(93.5)</td>
</tr>
<tr>
<td>Caste</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schedule caste</td>
<td>169(13.3)</td>
<td>93(10.7)</td>
</tr>
<tr>
<td>General caste</td>
<td>428(33.7)</td>
<td>307(35.3)</td>
</tr>
<tr>
<td>Other backward caste</td>
<td>311(24.5)</td>
<td>219(25.2)</td>
</tr>
<tr>
<td>Scheduled tribe</td>
<td>363(28.5)</td>
<td>251(28.8)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No formal schooling</td>
<td>357(28.1)</td>
<td>321(36.9)</td>
</tr>
<tr>
<td>Attended school any length of time</td>
<td>914(71.9)</td>
<td>549(28.8)</td>
</tr>
<tr>
<td>Socioeconomic characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Owns TV</td>
<td>567(44.6)</td>
<td>387(44.5)</td>
</tr>
<tr>
<td>Owns bicycle</td>
<td>1173(92.2)</td>
<td>687(78.9)</td>
</tr>
<tr>
<td>Owns house</td>
<td>958(75.4)</td>
<td>643(73.9)</td>
</tr>
<tr>
<td>Owns refrigerator</td>
<td>123(9.6)</td>
<td>83(905)</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------</td>
<td>---------</td>
</tr>
<tr>
<td>Roof material</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mud</td>
<td>622(48.9)</td>
<td>513(58.9)</td>
</tr>
<tr>
<td>Corrugated iron/asbestos sheet</td>
<td>242(19)</td>
<td>182(20.9)</td>
</tr>
<tr>
<td>cement/concrete</td>
<td>329(25.8)</td>
<td>107(12.3)</td>
</tr>
<tr>
<td>Other</td>
<td>78(6.1)</td>
<td>68(7.8)</td>
</tr>
<tr>
<td>Wall material</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mud/sand/dung</td>
<td>673(52.9)</td>
<td>478(54.9)</td>
</tr>
<tr>
<td>Mud bricks</td>
<td>127(9.9)</td>
<td>93(10.7)</td>
</tr>
<tr>
<td>Cement bricks</td>
<td>419(32.9)</td>
<td>267(30.7)</td>
</tr>
<tr>
<td>Other</td>
<td>52(4.1)</td>
<td>32(3.7)</td>
</tr>
<tr>
<td>Primary cooking fuel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wood</td>
<td>619(48.7)</td>
<td>387(44.5)</td>
</tr>
<tr>
<td>Charcoal</td>
<td>437(34.4)</td>
<td>279(32.1)</td>
</tr>
<tr>
<td>Gas</td>
<td>153(12.1)</td>
<td>136(15.6)</td>
</tr>
<tr>
<td>Other</td>
<td>62(4.9)</td>
<td>68(7.8)</td>
</tr>
<tr>
<td>Mode of delivery among pregnant women</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>n/a</td>
<td>586(67.3)</td>
</tr>
<tr>
<td>Caesarean</td>
<td>n/a</td>
<td>179(20.6)</td>
</tr>
<tr>
<td>Still Birth</td>
<td>n/a</td>
<td>105(12.1)</td>
</tr>
<tr>
<td>Birth Outcome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Term Delivery(≤36 weeks)</td>
<td>n/a</td>
<td>129(14.8)</td>
</tr>
<tr>
<td>Term Delivery (31-41 weeks)</td>
<td>n/a</td>
<td>623(71.6)</td>
</tr>
<tr>
<td>Post-Term Delivery (after 41 weeks)</td>
<td>n/a</td>
<td>118(13.5)</td>
</tr>
</tbody>
</table>

†Numbers may not add to sample size secondary to missing data.
* Defined as 3 or more pregnancies
** For ANC enrollees, gestational age assessed by fundal height. For DU enrollees, gestational age was assessed by Ballard score.
Table 2: Use of malaria prevention measures by pregnant women attending antenatal clinics and delivery units.

<table>
<thead>
<tr>
<th>Prevention measures utilized</th>
<th>Antenatal clinics n=1271 N (%)</th>
<th>Delivery units n=870 N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bed net in household</td>
<td>937(73.7)</td>
<td>643(73.9)</td>
</tr>
<tr>
<td>Insecticide-treated bed net in household</td>
<td>43(3.3)</td>
<td>21(2.4)</td>
</tr>
<tr>
<td>Sleeps under net most nights</td>
<td>873(68.6)</td>
<td>503(57.8)</td>
</tr>
<tr>
<td>Taken malaria prophylaxis in pregnancy</td>
<td>9(0.7)</td>
<td>3(0.3)</td>
</tr>
</tbody>
</table>

Table 3: Parasitaemia, reported fever, and anaemia among pregnant women attending antenatal clinics and delivery units

<table>
<thead>
<tr>
<th></th>
<th>Antenatal clinics n=1271 N (%)</th>
<th>Delivery units n=870 N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral Parasitaemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>68(5.4)</td>
<td>37(4.3)</td>
</tr>
<tr>
<td>Falciparum</td>
<td>3(0.23)</td>
<td>2(0.22)</td>
</tr>
<tr>
<td>Vivax</td>
<td>59(4.6)</td>
<td>32(3.67)</td>
</tr>
<tr>
<td>Mixed</td>
<td>6(0.47)</td>
<td>3(0.34)</td>
</tr>
<tr>
<td>By gravidity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primigravid</td>
<td>21/423(4.9)</td>
<td>11/338(3.2)</td>
</tr>
<tr>
<td>Secundigravid</td>
<td>38/578(6.6)</td>
<td>15/209(7.1)</td>
</tr>
<tr>
<td>Multigravid**</td>
<td>9/270(3.3)</td>
<td>11/323(3.4)</td>
</tr>
<tr>
<td>Report of fever within 1 week</td>
<td>167(13.1)</td>
<td>93(10.6)</td>
</tr>
<tr>
<td>Anaemia (Mild &amp; Moderate)</td>
<td>1076(84.6)</td>
<td>716(82.3)</td>
</tr>
<tr>
<td>Severe anaemia</td>
<td>173(13.6)</td>
<td>121(13.9)</td>
</tr>
</tbody>
</table>
Table 4: Univariate and multivariate analysis of predictors of peripheral parasitaemia among pregnant women attending antenatal clinics

<table>
<thead>
<tr>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral Parasitaemia</td>
<td>Relative Risk (95% CI)</td>
</tr>
<tr>
<td>% (Positive/Total)</td>
<td></td>
</tr>
<tr>
<td>First/second pregnancies</td>
<td>6.3% (64/1001)</td>
</tr>
<tr>
<td>Third or greater pregnancy</td>
<td>1.4% (2/270)</td>
</tr>
<tr>
<td>Age &lt; 20</td>
<td>7.2% (12/166)</td>
</tr>
<tr>
<td>Age ≥ 20</td>
<td>5.0% (56/1105)</td>
</tr>
<tr>
<td>Fever within past week</td>
<td>16.1% (27/167)</td>
</tr>
<tr>
<td>No fever within past week</td>
<td>3.7% (41/1104)</td>
</tr>
<tr>
<td>Bednet use*</td>
<td>7.6% (42/563)</td>
</tr>
<tr>
<td>No bednet use</td>
<td>6.7% (26/374)</td>
</tr>
<tr>
<td>Rural</td>
<td>7.1% (61/857)</td>
</tr>
<tr>
<td>Not rural</td>
<td>1.7% (7/414)</td>
</tr>
<tr>
<td>Tribal caste</td>
<td>6.3% (23/363)</td>
</tr>
<tr>
<td>Not tribal caste</td>
<td>4.9% (45/908)</td>
</tr>
<tr>
<td>No formal education</td>
<td>6.1% (22/357)</td>
</tr>
<tr>
<td>Formal education</td>
<td>5.0% (46/914)</td>
</tr>
<tr>
<td>Homeowner</td>
<td>5.9% (52/867)</td>
</tr>
<tr>
<td>Not homeowner</td>
<td>3.9% (16/404)</td>
</tr>
<tr>
<td>Mud walls</td>
<td>6.5% (44/673)</td>
</tr>
<tr>
<td>No mud walls</td>
<td>4.0% (24/598)</td>
</tr>
</tbody>
</table>

*Risk ratio adjusted for first/second pregnancies, fever within past week, rural locale, and * ITN use was not evaluated in this model since these were very rarely used as well as quite lesser awareness about ITN among women.
Table 5: Univariate and multivariate analysis of predictors of peripheral parasitaemia among women attending delivery units

<table>
<thead>
<tr>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral Parasitaemia</td>
<td>Relative Risk (95% CI)</td>
</tr>
<tr>
<td>% (Positive/Total)</td>
<td>Relative Risk (95% CI)</td>
</tr>
<tr>
<td>First/second pregnancies</td>
<td>5.8% (32/547)</td>
</tr>
<tr>
<td>Third or greater pregnancy</td>
<td>1.5% (5/323)</td>
</tr>
<tr>
<td>Age &lt;20</td>
<td>8.2% (28/109)</td>
</tr>
<tr>
<td>Age ≥ 20</td>
<td>3.6% (9/261)</td>
</tr>
<tr>
<td>Fever within past week</td>
<td>13.9% (13/93)</td>
</tr>
<tr>
<td>No fever within past week</td>
<td>3.1% (27/777)</td>
</tr>
<tr>
<td>Bednet use*</td>
<td>5.3% (27/503)</td>
</tr>
<tr>
<td>No bednet use</td>
<td>2.7% (10/367)</td>
</tr>
<tr>
<td>Rural</td>
<td>7.1% (29/405)</td>
</tr>
<tr>
<td>Not rural</td>
<td>1.7% (8/465)</td>
</tr>
<tr>
<td>Tribal caste</td>
<td>5.5% (14/251)</td>
</tr>
<tr>
<td>Not tribal caste</td>
<td>3.7% (23/619)</td>
</tr>
<tr>
<td>No formal education</td>
<td>5.3% (17/321)</td>
</tr>
<tr>
<td>Formal education</td>
<td>3.6% (20/549)</td>
</tr>
<tr>
<td>Homeowner</td>
<td>8.5% (28/327)</td>
</tr>
<tr>
<td>Not homeowner</td>
<td>1.6% (9/543)</td>
</tr>
<tr>
<td>Mud walls</td>
<td>5.6% (27/478)</td>
</tr>
<tr>
<td>No mud walls</td>
<td>2.5% (10/392)</td>
</tr>
</tbody>
</table>

†Risk ratio adjusted for fever within past week and age less than 20.
*ITN use was not evaluated in this model since these were very rarely used as well as quite lesser awareness about ITN among women.
CHAPTER 4
Introduction: -

Malaria is one of the destructive protozoan disease affecting human. Each year, around 0.5-3 million deaths are caused by *P. falciparum* species, mostly in sub-saharan Africa (268) while *P. vivax* the most rampant human malaria parasite (269) accounts for 130-390 million clinical episodes (270).

In the current scenario, malaria in pregnancy is one of the major priority medical challenges across the globe. Th1- Th2 cytokines are proteins of specific interest, since they are importantly involved in immunological responses pertaining to pregnancy and malaria infection and act as a prime messengers of adaptive immunity. In light of clinical evidence, it is indicated that pregnant women undergo immunological exchanges in
consensus with the debilitation of Th1 and strengthening of Th2 responses, thus establishing a successful pregnancy (271). In contrast, the augmentation of some forms of maternal cellular immunity is potentially detrimental for the fetal milieu. Cellular immunity mediated by effector cells and/or cytokines produced by them reflected remarkable harmful effects on the foetus. Malaria in pregnancy poses a significant risk to the mother, her fetus and the neonate (272). In due course of pregnancy, the immunity may be shifted towards Type 2 humoral defense mechanisms rather than towards Type 1 cellular responses, this primarily may be boonful for fetal well-being (273). Understanding the cytokine networks involving both pathology and protection may be significant when exploring the pathogenesis of malaria during pregnancy.

Although the pathophysiology of vivax malaria still remains poorly elucidated (274) adherence to endothelial cells (275) and sequestration of infected RBCs in deep vasculature (276) have been documented recently, while vivax malaria is becoming progressively common in South and South east Asia, Oceania and South America (277,278). For the effective clearance of parasitaemia, without inducing pathophysiology, a fine tuning between pro-inflammatory and anti-inflammatory cytokine response is required, whose timing and immensity is essential in the determination of malaria patient outcome (279). For the rapid effective clearance of *P. falciparum*, early production of TNF-alpha, IFN-gamma, IL-6, IL-12 is required (280,281,282).

Once parasitaemia is under control, regulatory cytokines such as IL-10 and transforming growth factor (TGF-beta) are crucial in reducing the risk of severe disease (283,284,285).

Cytokine responses have been thoroughly defined in *P. falciparum* infections in Africa and Oceania (282,285,286) but the process by which
immune responses of semi-immune subjects are regulated under low level exposure to various malaria parasite species is still undeterminate. Studies do suggest a shift towards regulatory cytokines in uncomplicated vivax malaria in Brazil (287). However, it is still poorly elucidated whether such a shift transcribes into milder clinical expositions and reduced risk of severe disease (288).

The current study aimed to explore the cytokine dynamics in malaria in pregnancy and healthy pregnant subjects following a cross-sectional study conducted at Hazaribag district of Jharkhand. In countries of malaria endemicity, subjects exposed to malaria infection acquire partially protective immunity to infection as they reach adulthood.

In difference, low level exposition to infection may lead to delayed immunity to plasmodium species and customarily in symptomatic disease (289).

In all endemic milieu, naturally acquired immunity is the outcome of the interaction of several host’s and parasite related factors (290) and among them the topic of the species and strain dependent immunity, appears very fundamental (291,292).

There is agreement on the crucial role of naturally acquired immunity to blood stage malaria in preventing acute disease and death due to *P. falciparum*. However, the exact mechanisms and significant determinants of such defense still need to be elucidated (293). Since, protective immunity against malaria is associated with the potency of transmission in moderate-high malaria transmission areas, maximum death due to malaria is distinguished in infants and young children, whilst semi-immune adults remain susceptible to asymptomatic parasitaemia, but protected against unsympathetic disease. In the absence of exposure, this protective immunity is lost after a few years. Acquired immunity against malaria is
also reduced in primigravidae, in fact they are vulnerable to severe *P. falciparum* disease due to placental malaria as they lack immunity to placenta specific cytoadherence proteins.

In case of malarial parasite afflicting non-pregnant subjects, both humoral and cell mediated immunity contributes to the acquisition of immune protection.

There is general consensus that cellular immune responses seem to play a crucial role in controlling the pre-erythrocytic stages of malaria infection, and antibodies do play a pivotal role to suppress blood stage infection. However, the process of acquisition of protective immunity against malaria still remains poorly understood, and no specific immune response documented so far positively correlate to clinical protection.

Collective evidence from previous studies suggests the importance of cell-mediated immune pathways in the adaptive response against malaria (294,295) and the central role played by CD4+ T cells in parasitaemia clearance and host survival (296,297). It is globally accepted that during the blood stage of infection, B cells and CD4+ T cells are activated and during the pre-erythrocytic or liver stage of the plasmodium life cycle CD8+ T cells are activated (298,299).

While the earlier reports on the immune response against *P. falciparum* have been debatable, the case of *P. vivax* infection has demonstrated more challenging milieu, primarily as a result of the absence of an in-vitro low term model of infection, and the common co-existence with *P. falciparum* in areas of high endemicity (300). The study aimed to explore the cytokine profiles in systemic circulation of malaria in pregnancy subjects attending ANC of Sadar Hospital, Hazaribag so as to surplus the ongoing data on the pathogenesis of malaria in this locality.
Although aspects of the cytokine imbalance have been globally investigated in malaria in pregnancy, no data existed concerning the cytokine dynamics pertaining to malaria in pregnancy in this locality.

As far as our knowledge is concern, this work is the first systematic investigation pertaining to malaria in pregnancy and cytokine dynamics in this locality, which prompted us to carry out this investigation.

In order to understand the immunological mechanisms occurring in malaria in pregnancy subjects, we probed the levels of Th1 and Th2 cytokines in peripheral blood of pregnant subjects enrolled in a cross-sectional study conducted at Hazaribag district of Jharkhand.

There is a significant gap in the knowledge of the immunity against \textit{P. vivax} in non-pregnant and pregnant subjects compared to \textit{P. falciparum}. African countries accounts for most of the cases of gestational malaria, in which malaria transmission is reported in 43 countries.

The aim of this research is to present and discuss the cytokine dynamics in malaria in pregnancy and healthy pregnant subjects with emphasis on research carried out at Hazaribag district of Jharkhand, in order to set up an interpretation for the studies pertaining to pregnancy and malaria that are recently a hot topic of research.

**Dynamics of gestational and placental malaria**

Defining gestational and placental malaria differs with respect to authors and techniques applied for establishing diagnosis. According to the definition of WHO, gestational malaria is the presence of parasitaemia in placenta or peripheral blood (301) and thick smear examination is the advocated test for diagnosis in endemic areas (302).

The term pregnancy associated malaria is generally defined as an infection or disease caused by \textit{plasmodium} species during pregnancy with the
concurring of three components: gestational malaria, placental malaria and congenital malaria.

In African mothers residing in high endemic areas, parasitaemia by *P. falciparum* seem to outstretch a peak between 13-16 of gestation (303,304). In case of *P. vivax* infections during pregnancy, mortality is seldom seen, but it is associated with multiple relapses, anaemia, abortion and a reduced birth weight in pregnant women with malaria (305, 306-308). Pregnant women have an increased probability to experience relapses than non-pregnant ones (307).

*P. vivax* sequestration has been advocated (309) and current record stipulated disproportional organ-specific and peripheral blood parasitaemia (310). No reports have addressed the issue pertaining to the dynamics of infection throughout pregnancy. Current findings confirmed the conception that diagnosis and treatment of pregnancy related malaria during early gestation arrests miscarriage both in *P. vivax* and *P. falciparum* infections (300,306). In most African countries, *P. falciparum* is very prevailing, almost unique, as a result pregnant women customarily experience malaria due to this species. The case is different in most Asian and American endemic regions, where in general *P. vivax* is prevailing (60-70%) and women experiences infection by both *P. vivax* and *P. falciparum*.

From the recent review of malaria in the Asian-pacific region, it has been documented that a median proportion of pregnant women infected with malaria in antenatal clinics of 15.3% and in delivery rooms of 8.1% shown by thick smear evaluation test (308). Moreover, the median proportion of documented placental parasitaemia was 11%. The same study demonstrated that *P. vivax* accounts for a median of 21.1% of malaria infections at ANC.
In South East Asian region, it has been demonstrated that *P. vivax* infection have a protective effect against successive event and severity of *P. falciparum* malaria in Thailand (307,311).

Furthermore, it has been postulated that a critical event of *P. falciparum* could activate a relapse of a formerly acquire *P. vivax* infection (312). Therefore, as the probability of occurance of *P. falciparum* increases, the number of *P. vivax* incident caused by relapses will finally also increase.

Furthermore, in order to establish a true burden of malaria in pregnancy, a longitudinal set up is required.

Altogether, in the light of collective evidence, studies confirm that *P. vivax* malaria during pregnancy is a problem in many endemic countries outside Africa and its consequences in both mother and foetus remain poorly elucidated (313-316).

In order to acquire tolerance towards paternal antigens expressed on foetal cells, the immune system during pregnancy is modified (317).

In this process, cytokines work in a coordinated manner either at the maternal-foetal interface or systemically (318,319). Evidence of such process is distinguished in the mother as cytokine dynamics could be remarkably noticed at different trimesters of pregnancy.

In the peripheral blood circulation of healthy pregnant women, the production of IL-10, IL-4, IL-6 and IL-13 progressively increases, while serum levels of most Th1 type cytokines (IL-1 alpha, IL- beta, IL-2, IL-12, IFN-gamma) remarkably decrease in the third trimester as compared to first trimester of pregnancy (320,321,322).

Production of IL-2, IFN-gamma, TNF-alpha and IL-12 by Th1 cells are believed to drive tissue damage in some inflammatory diseases including malaria (323).
Production of IL-4, IL-5, IL-13 by Th2 cells mediates B cell activation and antibody production and it is well established that these two pathways reciprocally impede each other (324-326).

Therefore, the result of Th2 biased immune response during normal pregnancy involves suppression of cell mediated Th1 cytokines immunity and amplification of Th2 biased humoral responses (327).

Furthermore, perturbances in the cytokine networks can have an adverse pregnancy outcomes including spontaneous abortion, preterm labour, pre-eclampsia and intrauterine growth restriction (328,329). Such is the manifestation of gestational malaria, in which pro-inflammatory rank has been established and bestow to describe the detrimental sequel of *P. falciparum* infection in mother and foetus (330).

It is well established that the process of accession of immunity against malaria during pregnancy only starts when the women residing in an endemic area becomes pregnant, since formerly acquired immunity decline to defend against maternal and placental infection (331,332). So, some degree of protection might be achieved after repeated exposition to particular parasite throughout several pregnancies.

A strong pro-inflammatory milieu is observed in an infected placenta which focused at controlling parasites’s multiplication. Despite the immense importance of cell mediated responses in the immunity against *Plasmodium* species their overproduction may constitute a threat to successful pregnancy. Since, the source of pro-inflammatory cytokines might both be the placental and fetal cells exposed to antigens, they may imperil pregnancy (333, 334).

The interplay between inflammation, pregnancy and malaria still requires further exploration to set up a framework of hypothesis in this area of
research. Noteworthy, the complicated interactions between the host and the parasite in pregnant subjects are the hot topic of research.

2. Materials and Methods

Patients

After obtaining the informed consent from the study participants, serum sample of 50 malaria in pregnancy subjects and 50 Healthy pregnant women were assayed. About 5 ml of peripheral blood were taken into sterile glass tubes and immediately centrifuged at 3500 rpm for 10 min at 4 °C, 0.5 ml aliquots of the supernatant were immediately frozen in polypropylene tubes at -70°C and stored until assay. There were no freeze thaw cycles. Commercial enzyme-linked immune assays were used as recommended by the manufacturer for the estimation of serum IL-1 beta, IL-6, IL-8, IL-10, TNF-alpha. Each plate included standard of human cytokine run in parallel with samples. All samples were run in duplicates and the mean values was used in all analyses.

Statistical analysis

All values were expressed as mean±S.D. Statistical analysis were done by computing data in SPSS. Unpaired t-test was used to test the mean difference between the two groups.

Criteria for inclusion and Exclusion: -

Inclusion criteria was good health, no history of serious disease, no history of infection and autoimmune diseases. Exclusion criteria were withdrawal of informed consent, auto immune disease, Infectious disease.
Ethical Clearance: -

The study protocol was approved by Institutional review Board of Vinoba Bhave University.

Results: -

A significant decrease in IL-6 concentration has been observed in Malaria in pregnancy (2.32±0.015 ng/ml) as compared to Healthy pregnant women (2.63±0.012 ng/ml). (p˂.0001).

Depressed level of IL-8 was seen in sera of Malaria in pregnancy (1.68±0.020 ng/ml) than their healthy counterparts (1.74±0.028 ng/ml) and the difference was statistically significant (p˂.0001).

TNF-alpha was significantly elevated in MIP (0.90±0.01 ng/ml) as compared to their counterparts HPW (0.68±0.015 ng/ml). A significant increase in IL-1beta has been noted in MIP (1.88±0.141 ng/ml) as compared to HPW (1.62±0.015 ng/ml) (p˂.0001).

Increase in the level of IL-10 has been observed in MIP (0.74±0.010 ng/ml) as compared to their healthy counterparts (0.54±0.012 ng/ml) and the result was statistically significant (p˂.0001).

Discussion: -

Successful pregnancy in vertebrates is the result of the potency of the maternal immune system to retain the fetal allograft (335). Cellular response is the principle mediator of allograft rejection (336) and the augmentation of cell mediated immunity is associated with the secretion of the type 1 cytokines IL-2, IFN-gamma and IL-12 (337-341). Previous studies validate the concept that successful pregnancy in mice is a Th2 biased phenomenon (342,343) and that a remarkable increase in the concentrations of type 1 cytokines have an association with spontaneous
abortions and impaired fetal development in mice (342,343) and humans (344-346).

We analysed the cytokine profile in healthy and malarous pregnant subject, in order to establish whether alteration in cytokine profiles are subject to normal pregnancy or whether malaria is associated with specific cytokine patterns.

In the current investigation, the concentrations of cytokines are used both for the assessment of pathology and protection. Cytokines have been known to act as biomarkers for the assessment of pathophysiological processes (347) and may function as biochemical second messenger (348).

Among these IL-10 is known for its anti-inflammatory role and IL-6, IL-8, IL-1beta, TNF-alpha have pro-inflammatory effects. In the current study, we reported a predominant increase in the concentration of anti-inflammatory cytokine (IL-10) in MIP as compared to its healthy counterparts. A positive correlation between MIP and IL-10 concentration enable us to infer that an increased anti-inflammatory response may limit the threshold required for the clearance of parasitaemia. Increased levels of IL-10 may promote parasite multiplication by inhibiting parasite-killing effector mechanisms in humans (349) and mice (350).

In the current investigation, an elevated level of IL-6 and IL-8 suggests the role of these cytokines in the immune-pathogenesis of malaria infection in the peripheral circulation of pregnant women in the studied population.

Increased level of TNF-alpha and IL-1 beta in the peripheral circulation of MIP subjects as compared to its healthy counterparts implicates their role in immuno-protection and in the clearance of parasitaemia.

This research focused to explore the cytokine profiles among MIP and healthy pregnant subjects attending ANC of Sadar Hospital, Hazaribag. This is a cross sectional study of the plasma levels of cytokines,
measurements that are still not recorded in the literature for this particular population.

Since, we measured cytokine at a single time point, it's hard to establish the precise interplay of cytokines and its role in pathology and protection. For the inference of causal relationship, a longitudinal set-up is required; however, our data do reflect the trend of cytokine networks but fail to depict the sequence of events from parasite inoculation to the onset of disease.

**Conclusion:**

A lot of evidence gathered so far modified our perception of malaria and the role played by the immune system in both protection and pathogenesis. Fine tuning between both pro- and anti-inflammatory cytokine is required to ensure adequate protection.

For the effective clearance of the parasitaemia, an early strong pro-inflammatory cytokine response is required, but during the later stage of an infection, there comes the role of anti-inflammatory response to counterbalance the effect of pro-inflammatory response and thus conferring protection.

Complex networking prevails in the pathogenesis of malaria in which a common outcome might be reached by several pathways. Routine detection of some of the cytokines may be significant to diagnose prognose the various clinical conditions that might have distinct immunological features.
Table 6: This table illustrates the cytokine profile of malaria in pregnancy compared to healthy pregnant subjects attending ANC of Sadar Hospital, Hazaribag.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Malaria in pregnancy (MIP)</th>
<th>Healthy Pregnant Women (HPW)</th>
<th>P-value (Sig 2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>2.32±0.015</td>
<td>2.63±0.012</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.68±0.020</td>
<td>1.74±0.028</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>0.90±0.01</td>
<td>0.68±0.020</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>IL-1beta</td>
<td>1.88±0.141</td>
<td>1.62±0.015</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.74±0.010</td>
<td>0.54±0.012</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

All values are expressed as mean±S.D.
CHAPTER 5
CYTOKINE PROFILING IN PREGNANT AND NON-PREGNANT SUBJECTS.

Introduction: -

Cytokines are the proteins produced in an autocrine or paracrine function, bind to specific receptors initiating a cascade of reactions on different targets having beneficial or harmful effects and its imbalance has been accused for many pathological disorders. Microbial infection, certain auto immune diseases have been linked to over or under production of cytokines which are produced in response to various immune stimuli. The naturally occurring immune reaction in an organism is developed during the gestational period where, inspite of the presence of a semi-allogeneic graft, maternal immune response is regulated to support the fetal allograft. The success of an embryo development is allocated to the important involvement of cytokines where some have been designated critical and others deleterious to fetal growth. TH cells are the major
producers of cytokines, and the balance of Th1 versus Th2 cytokines describes the welfare of an organism (351, 352) Pregnancy is the natural phenomenon of an immune reaction occurring for a determined time course antagonizing the principles of graft rejection. The semi or allogeneic fetal components emerging in the privileged site of uterus, not only evades maternal immune attack but are assisted by the maternal immune system. Cytokine plays an important role in the maintenance of pregnancy, where a successful pregnancy is correlated to the production of Th2 cytokines and fetal rejection to Th1 cytokines. Although the protective role of the Th2 cytokine IL-10 during mid-gestation (353) as well as the deleterious effects of Th1 cytokines IL-2 (354) TNF-alpha (355,356,357) and IFN-gamma (355,358) have been evidenced in mice, other studies documented that each cytokine explicits a definite pattern of expression each day of pregnancy in mice (359) These data are an indicative of the fact that inspite of the beneficial or harmful effect of a cytokine during a specific time course of the gestational cycle, a determined up or down regulation of these factors must be followed, a pattern on which the success of pregnancy depends. Thus, it is essential to explore the physiological levels of cytokines during the time course of pregnancy, which may eventually have a prognostic role for pregnancy outcomes.

With the advancement of pregnancy, a maternal shift is observed, away from a type 2-biased immune response towards an inflammatory response. During pregnancy, the female immune system encounters an exquisite stabilizing act. On one hand, it must evolve tolerance to paternal antigens to arrest fatal immunologic charges against the fetus. On the other hand, it must sustain the potential to combat infections from a diverse variety of environmental pathogens. Perturbances in this balance may lead to detrimental consequences, including preterm birth and death of fetus. The normal maternal immune response to pregnancy is remarkably a process experiencing changes, in which the maternal pro/anti-inflammatory profile
changes at different phases of gestation (360, 361, 362). Inspite of this, a thorough, longitudinal immunologic profile of normal pregnancy has not been documented. Such evaluation is crucial for understanding the response to specific infectious and immunologic diseases that manifests disproportionately negative outcomes during pregnancy, such as influenza (363) and ulcerative colitis (364). The inflammatory cytokines, IL-1 beta, IL-6 and IL-8 are intricated in the maintenance of trophoblast in early pregnancy. IL-1 beta has been propounded as the principle cytokine of the inflammatory response (365) which effectively stimulate IL-6 and IL-8 among other cytokines. IL-8 is a chemotactic cytokine moderating the rupture of the membranes and cervical ripening (366) along with IL-6, it plays a crucial role in preterm cervical remodeling (367,368). Serum IL-1 beta, IL-6 and IL-8 seem to play an important role during pregnancy (369,370,371,372) towards term and at delivery (373,374,375) remarkably with increased levels in labour (370,376,366). The current research focusses to evaluate and establish the cytokine profile across trimesters in pregnant subjects and non-pregnant subjects of the participants attending antenatal clinics of Sadar Hospital Hazaribag. To the best of our knowledge, such immune modelling profile have not been investigated in this locality. So, this study is an attempt to establish physiological concentrations of these cytokines at certain time course, where utilizing this knowledge and further research may be useful for therapeutic interventions in the future.

**Materials and Methods:**

A cross sectional study was conducted at Sadar Hospital Hazaribag involving 200 Healthy Pregnant women (90 in 1st Trimester, 45 in 2nd Trimester, 65 in 3rd Trimester) and 100 non-pregnant subjects following institutional review board approval. Participants were enrolled in the study protocol after obtaining their written consent, if the clinical examination
was normal. Standardized questionnaires were administered for obtaining history and general status of the study participants.

Criteria for inclusion and Exclusion: -

Inclusion criteria was good health, no history of serious disease, no history of infection and autoimmune diseases. Exclusion criteria were withdrawal of informed consent, auto immune disease, Infectious disease.

Cytokines: -

About 5 ml of venous blood was drawn into sterile glass tubes and centrifuged at 3500 rpm for 10 min at 4˚C, 0.5 ml aliquots of the supernatant were frozen in polypropylene tubes at -70˚C and stored until assay. Commercial enzyme linked immunoassays were used according to the manufacturers recommendations to assess IL-1 beta, IL-6, IL-8, TNF-alpha, IL-10 (BD Bioscences kit). Before use, all reagents and samples were brought to room temperature. Frequent freeze thaw cycles were avoided. Measurement of serum cytokine concentrations was done using an ELISA method as specified by the supplier at test and reference wavelengths of 450 nm and 550 nm respectively. All samples were run in duplicates and the mean value was used for analysis.

Statistical analysis: -

Statistical analysis was performed using SPSS Version 21. Data were analysed by one-way analysis of variance. Post hoc tukey’s test was performed for comparison of differences between means of non-pregnant subject and pregnant subjects across trimesters and labour. All values were expressed as mean±S.D. The mean difference is significant at the 0.0001 level.
**Results:**

Cytokine concentrations in the serum are the indicators of pathologic state of an organism and may, in many cases, have a prognostic nature for therapeutic interventions. In this research, we focused our interest to human pregnancy where the rates of fetal rejection extraordinarily increase, we assessed the physiological levels of different cytokines across trimesters in pregnant and non-pregnant subjects.

The objective of this work was to establish the physiological concentration of cytokine in pregnant and non-pregnant subjects. In view of this investigation, we discovered, statistically significant differences between the non-pregnant and pregnant subjects for IL-1 beta (1\textsuperscript{st} trimester vs non-pregnant (p<0.0001), 2\textsuperscript{nd} trimester vs non-pregnant (p<0.0001), 3\textsuperscript{rd} trimester vs non-pregnant (p<0.0001), labour vs non-pregnant (p<0.0001). Strikingly significant peak value of IL-1 beta was observed in 3\textsuperscript{rd} trimester and labour when compared to non-pregnant subject.

For IL-6, significant differences between non-pregnant subject and pregnant subjects across trimesters were observed (p<0.0001). Strikingly, increased value of IL-6 was observed in 2\textsuperscript{nd} trimester, 3\textsuperscript{rd} trimester and labour as compared to non-pregnant subjects. In respect to IL-8, significant differences between non-pregnant and pregnant subjects were noted in 2\textsuperscript{nd} trimester, 3\textsuperscript{rd} trimester and labour (p<0.0001). Statistically peak values were obtained for 3\textsuperscript{rd} trimester and labour as compared to non-pregnant subject (p<0.0001). However, no significant correlation was observed when comparing non-pregnant subject to 2\textsuperscript{nd} trimester (p=0.004).

Similarly, findings in context to TNF-alpha and IL-10, statistically significant differences between non-pregnant subjects and pregnant subjects in 2\textsuperscript{nd} trimester, 3\textsuperscript{rd} trimester and labour existed (p<0.0001). Noteworthy, when comparing non-pregnant subject with pregnancy in the 1\textsuperscript{st} trimester, did not show any significant result (p=0.870). TNF-alpha
presented a stable production profile at different stages of pregnancy and labour. Statistically significant elevated values of IL-10 was noted in 3\textsuperscript{rd} trimester and labour thus reflecting its counterregulatory role.

**Discussion:** -

The investigation carried out allocates a preliminary normative data for the cytokine profile in pregnant and non-pregnant subjects. It has been propounded that pro-inflammatory cytokines (e.g. IL-6) are important in triggering birth (377,378). Our findings indicate that the 3\textsuperscript{rd} trimester of pregnancy and labour is marked by elevated inflammatory (e.g. IL-1 beta, IL-6, IL-8) cytokine environment. Our results also reflected the peak production of IL-10 during 3\textsuperscript{rd} trimester and labour, an agent known to play a defensive role during pregnancy (352,379). Above finding corroborates with other reports of systemic immune activation as well as counterregulation at the end of pregnancy (362,380,381,382). Increasingly IL-1 beta level may principally be associated to production from placental tissues (383,384) or mononuclear phagocytes (385,386) in late gestation. Since, our study is a single time point, the exact immunologic mechanisms accountable for this shift cannot be estimated by our current investigation. Furthermore, the changes in the concentrations of the cytokines pinpointed in our investigation could probably be due to changes in peripheral blood cellular composition. Further investigations will be mandatory to validate whether placental tissues, circulating leukocytes, stromal cells, or other sources also accord for the changes indicated in our results. Some studies documented decreasing IL-1 beta levels throughout gestation (387) contradictory to our investigation in which IL-1 beta peak has been observed in 3\textsuperscript{rd} trimester and labour. Our report confirmed increase in IL-1 beta, IL-6, IL-8 notably during 3\textsuperscript{rd} trimester and labour indicating that these cytokines do play a major role during pregnancy (369,370,371,372) towards term and at delivery (373,374,375) remarkably with peak levels in
labour (370,376,366). In view of current investigation, our study documents that the inflammatory cytokines IL-1 beta, IL-6 and IL-8 already exists in maternal serum in early pregnancy but undergo little variation subsequently until term. High cytokine concentration (IL-1 beta, IL-6, IL-8) accounted in present research at the 3rd trimester and labour reflects that labour resembles an inflammatory process. Peak value of IL-8 in 3rd trimester and labour observed in our study demonstrates that IL-8 has been proposed as the final common step in prostaglandin and anti progestagen action in parturition (388). The increase in the concentration of IL-6 and IL-8 in preterm (389,390) and term labor (373) is still controversial (391). Some of the earlier studies contradicts our findings, documenting the decrease of TNF-alpha, IL-1beta and IL-6 across trimesters in samples of healthy pregnant women collected during each trimester (387). Individual heterogeneity and lack of longitudinal analysis in our study may be accountable for some differences with prior studies.

Our study corroborates with the report (370) which shows an increase of IL-6 and IL-8 during normal term labour (370). Our current investigations validate the notion that IL-1beta, IL-6, IL-8 do play a role in the maintenance of human pregnancy. They reflect that increased cytokine levels are the function of labor. In respect to TNF-alpha level in our present finding, a balanced cytokine profile has been accorded for the same, an agent accused for pregnancy failure (358,356,357). IL-10, shown to play a protective function during pregnancy (353,379) recorded a peak value in 3rd trimester and labour. One mechanism by which the fetus maintains its immunological advantage in the uterus is to firmly regulate the cytokine concentration at the maternal-fetal interface (392). Although some physiological roles of pro-inflammatory cytokines at the maternal-fetal interface have been explained in association with the growth of the placenta and decidua (393) much of the literature authenticated the concept that excessive production of proinflammatory cytokines such as IL-1 beta,
TNF-alpha and IFN-gamma at the maternal fetal interface is detrimental to pregnancy. IL-10 is plausibly an important counterregulator because it down regulates the production of pro-inflammatory cytokines by other cells and a number of studies suggested its production at the maternal-fetal interface (394,395,396). Earlier studies documented that successful pregnancy is predominantly a type 2 immune response-biased phenomenon (397,398,399). In view of current investigation, with the advancement in the stages of pregnancy, a maternal shift away from a type 2-biased immune responses and towards an inflammatory response is observed. Furthermore, anti-inflammatory or type 2 immune response do play a protective role during pregnancy as evidenced by the peak value of IL-10 in 3rd trimester. In view of the collective evidence, further refinement is the need to describe pregnancy in terms of type 2 immune responses, both in the overall description of key cytokines as well as in the time related dynamics.

Our study is limited by lacking the longitudinal analysis of the sample. Our results require further authentication by longitudinal analysis which could better describe individual maternal immune variation.
Table 7 – Cytokine profile in Non pregnant women compared to Pregnant women across trimesters.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Non Pregnant Women</th>
<th>1st Trimester</th>
<th>2nd Trimester</th>
<th>3rd Trimester</th>
<th>Labour</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 beta</td>
<td>1.66±0.018</td>
<td>1.65±0.017*</td>
<td>1.45±0.015*</td>
<td>1.86±0.023*</td>
<td>1.86±0.0091*</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.66±0.020</td>
<td>2.32±0.143*</td>
<td>2.84±0.014*</td>
<td>2.85±0.018*</td>
<td>2.82±0.014*</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.77±0.022</td>
<td>1.75±0.012ns</td>
<td>1.75±0.013*</td>
<td>1.92±0.042*</td>
<td>1.98±0.010*</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>0.78±0.013</td>
<td>0.77±0.012ns</td>
<td>0.67±0.037*</td>
<td>0.69±0.015*</td>
<td>0.69±0.008*</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.78±0.013</td>
<td>0.77±0.013ns</td>
<td>0.76±0.017*</td>
<td>0.89±0.014*</td>
<td>1.008±0.046*</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

P* Significant when compared to non-pregnant subject.

Pns Non-significant when compared to non-pregnant subject.

Results were significant at P<0.0001.
CHAPTER - 6
Haematological parameters and its association with malaria in pregnant and non-pregnant subjects at Hazaribag district of Jharkhand.

1. Introduction

Over 50 million women are manifested to the risk of malaria in pregnancy every year. Malaria in pregnancy leads to considerable maternal and especially fetal and infant morbidity, causing 75,000-2,000,000 infant deaths each year (400). Both *Plasmodium falciparum* and *Plasmodium vivax* infections can obtrude untoward pregnancy consequences, including maternal anaemia and low birth weight due to preterm delivery and fetal growth restriction, differing in mechanisms (401).

Pregnant women are at an increased threat of malaria infection compared to non-pregnant women, and the risk is greatest in first and second pregnancy. Susceptibility to pregnancy associated malaria plausibly indicate a combination of immunological (402) and hormonal changes related to pregnancy. Malaria is detrimental to both the mother and foetus. Parasite densities are higher in pregnant women compared to non-pregnant
counterparts. Studies do report that the propensity to limit parasite replication is impaired in pregnancy.

Malaria is most recurrent in first pregnancy (403), peaking between 13 and 16 weeks (404) and repulsing towards term. In areas with low malaria transmission, maternal disease is often severe due to lack of pre-existing immunity (405). Malaria in pregnancy is distinctive because of the sequestration of parasites in the placenta where infection is extremely heavy. Although some haematological changes associated with malaria are generally acknowledged, there are contradictory reports on variation in some haematological parameters. For instance, malaria has been associated with anaemia and non-anaemia, mild and severe thrombocytopenia, leucopenia and leukocytosis (406). Haematological changes associated with malaria infection in pregnancy have not been well documented in the district under investigation. As per our knowledge is concerned, such epidemiological profile have not been previously investigated in this locality, which prompted us to explore this in the concerned population.

To address this enormous burden of malaria, we have two proven tools. The use of insecticide treated nets and intermittent preventive treatment in pregnancy, using regular treatment doses of the antimalarial sulphadoxine-pyrimethamine (SP) has been shown to decrease peripheral and placental parasitaemia and to increase maternal Hb and infant birth weight, especially in primi and secundigravidae (407). The development and assessment of programs to prevent malaria in pregnancy can be eased by a better understanding of the pathogenesis of malaria. Considering the lack of scientific investigations on the effects of malaria parasite on haematological profile of pregnant women, the current investigation was drafted to determine the effects of malaria parasite on HCT, WBC, RBC count, Hb estimation and PLT count.
2. Materials and methods

2.1 Study design
This is a cross sectional study involving data collection from pregnant women attending ANC and non-pregnant women attending the outpatient department.

2.2 Study site
The study was conducted at Hazaribag district of Jharkhand having perennial malaria transmission with a yearly average slide positivity rate SPR for symptomatic individuals of 7.3% over last three years with *P. falciparum* constituting of about 14% of the cases (408). Hazaribag is a highly endemic area of *P. falciparum* and *P. vivax* infection with a peak occurrence from July to October.

2.3 Study subjects
The study population consisted of pregnant women attending the ANC of Sadar Hospital, Hazaribag and non-pregnant women attending the outpatient department of the health clinics served as control. The groups constituted of pregnant women with malaria (50), pregnant women without malaria (50) and non-pregnant with malaria (50). Inclusion in the study protocol were of pregnant women and non-pregnant women who consented to the study, participants with good health. Exclusion criteria included women who did not consent to the study and pregnant women who had HIV and other infectious disease history which could alter the haematological indices. Questionnaires were administered to obtain the clinical correlates of the enrolled subjects.
2.4 Ethical clearance

The study protocol was carried out in accordance to the Vinoba Bhave University, Hazaribag, human ethical guidelines, as illustrated in the guidelines of the Medical Ethics Committee, Ministry of Health, India.

2.4 Sample collection

A sample of venous blood (5ml) was collected from each participant after their informed consent with minimal stasis from the antecubital vein using a dry, sterile disposable syringe. The blood was collected into EDTA coated tubes.

2.5

Malaria parasites detection was carried out by microscopic examination of thin and thick blood films with 3% Giemsa. Haematocrit, WBC, RBC count, Hb concentration, platelet count were determined using an automated analyzer.

2.6 Statistical analysis

All data were expressed as mean ± S.D. Data were analyzed using SPSS. Parameters comparisons were done using Student’s t test. A P-value of < 0.05 was considered significant.

3 Results

Table 1 shows the haematological characteristics of pregnant women with malaria as compared to non-pregnant women with malaria group. Mean WBC count was higher in pregnant women with malaria (6.7±0.33) group as compared to non-pregnant with malaria group (5.7±0.33). Mean haemoglobin concentration in pregnant women with malaria (8.04±0.375) group was lower as compared to non-pregnant women with malaria (9.4±3.15) group. Mean RBC count was lower in
pregnant women with malaria (3.13±0.192) as compared to non-pregnant women with malaria (3.62±0.189) group. Mean HCT was lower in pregnant women with malaria (32.1±0.190) as compared to non-pregnant women with malaria (32.8±0.187). Mean PLT count was lower in non-pregnant women with malaria (145.78±3.59) as compared to pregnant women with malaria group (114.94±5.08).
Table 8: - Haematological characteristics of pregnant women with malaria and non-pregnant women with malaria group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pregnant with malaria</th>
<th>Non-Pregnant with malaria</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC count,×10⁹</td>
<td>6.7±0.33</td>
<td>5.7±0.33</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Hb(g/dL)</td>
<td>8.04±0.375</td>
<td>9.4±0.315</td>
<td>P=0.832</td>
</tr>
<tr>
<td>RBC(mmol/L)</td>
<td>3.13±0.192</td>
<td>3.62±0.189</td>
<td>P=0.909</td>
</tr>
<tr>
<td>HCT,%</td>
<td>32.1±0.190</td>
<td>32.8±0.187</td>
<td>P=0.847</td>
</tr>
<tr>
<td>PLT count,×10⁹</td>
<td>114.94±5.08</td>
<td>145.78±3.59</td>
<td>P=0.131</td>
</tr>
</tbody>
</table>

Table 8: - represents the haematological characteristics of pregnant women with malaria as compared to pregnant women without malaria group. Mean WBC count was higher in pregnant women with malaria (6.7±0.33) as compared to pregnant women without malaria (3.6±0.25) group. Mean Hb concentration in pregnant women with malaria (8.04±0.375) was lower as compared to pregnant women without malaria (11.4±0.263). Mean RBC count was lower in pregnant women with malaria (3.13±0.192) as compared to pregnant women without malaria group. HCT concentration was significantly lower in pregnant women with malaria (32.1±0.190) as compared to pregnant women without malaria (35.17±1.44) group. PLT count was lower in pregnant women with malaria (114.94±5.08) group as compared to pregnant women without malaria (215.36±4.78) group.
Table 9: - Haematological characteristics of pregnant women with malaria and pregnant women without malaria groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pregnant with malaria</th>
<th>Pregnant without malaria</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC count, ×10^9</td>
<td>6.7±0.33</td>
<td>3.6±0.25</td>
<td>0.288</td>
</tr>
<tr>
<td>Hb(g/dL)</td>
<td>8.04±0.375</td>
<td>11.4±0.263</td>
<td>0.842</td>
</tr>
<tr>
<td>RBC(mmol/L)</td>
<td>3.13±0.192</td>
<td>3.83±0.191</td>
<td>0.877</td>
</tr>
<tr>
<td>HCT, %</td>
<td>32.1±0.190</td>
<td>35.17±1.44</td>
<td>0.000</td>
</tr>
<tr>
<td>PLT count, ×10^9</td>
<td>114.94±5.08</td>
<td>215.36±4.68</td>
<td>0.452</td>
</tr>
</tbody>
</table>

Table 9: - Shows haematological characteristics of non-pregnant women with malaria as compared to pregnant women without malaria. Mean WBC count was higher in non-pregnant women with malaria (5.7±0.33) as compared to pregnant women without malaria (3.6±0.25) group. Mean Hb concentration was lower in non-pregnant women with malaria (9.4±0.315) group as compared to pregnant women without malaria (11.4±0.263) group. Mean RBC count was lower in non-pregnant women with malaria (3.62±0.189) group as compared to pregnant women without malaria (3.83±0.191) group. Mean HCT concentration was significantly lower in non-pregnant with malaria (32.8±0.187) as compared to pregnant women without malaria (35.17±1.44) group. Mean PLT count was lower in non-pregnant women with malaria (145.78±3.59) group as compared to pregnant women without malaria (215.36±4.68) group.
Table 10: - Haematological characteristics of non-pregnant women with malaria and pregnant women without malaria group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-pregnant with malaria</th>
<th>Pregnant without malaria</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC count, ×10⁹</td>
<td>5.7±0.33</td>
<td>3.6±0.25</td>
<td>0.288</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>9.4±0.315</td>
<td>11.4±0.263</td>
<td>0.959</td>
</tr>
<tr>
<td>RBC (mmol/L)</td>
<td>3.62±0.189</td>
<td>3.83±0.191</td>
<td>0.966</td>
</tr>
<tr>
<td>HCT, %</td>
<td>32.8±0.187</td>
<td>35.17±1.44</td>
<td>0.000</td>
</tr>
<tr>
<td>PLT count,×10⁹</td>
<td>145.78±3.59</td>
<td>215.36±4.68</td>
<td>0.507</td>
</tr>
</tbody>
</table>
4 Discussion

Malaria in pregnancy is a serious challenge encountered globally which should be checked upon. Pregnant women are more susceptible to malaria because pregnancy leads to immune suppression and the mother is increasingly reliant on humoral immunity for protection, thus making her more susceptible to malaria (409). Blood is a good indicator to determine the physiological status of an individual and the assessment of haematological parameters can be used to determine the extent of deleterious effects of foreign agents including malaria parasite (410).

The current study corroborates with the finding (411) which explored that malaria parasite led to the decrease in Haemoglobin concentration, RBC count but these decrease were not statistically significant and less likely to lead to severe anaemia. Anaemia in malaria is due to increase in haemolysis and decrease in the rate of production of RBCs and accelerated removal of both parasitized RBC and unparasitized RBC (412). Other factors which accord for anemia in malaria include increased RBC deformity, splenic phagocytosis and or pooling (413). The outcome of Hb result infers the devastating effects of cohabitation of malaria with pregnancy which induced anaemia in the pregnant women with malaria group as defined by the WHO. The HCT concentration in the pregnant women without malaria group was significantly higher when compared to the two studied group investigated. This study manifested that malaria infection was strongly correlated with reduction in HCT and further devastating effect of combination of pregnancy and malaria also adds to the decrease in HCT concentration. Strikingly, current finding corroborates with other reports (414) which reported that decrease in Hb and HCT concentration were common findings on pregnancy and attributed this to increased plasma volume combined with poor iron intake.
Matthews et al (415) recorded that platelet count remains in the normal pregnant range in most women during uncomplicated pregnancies and Verdy et al (416) reported that mean platelet count of pregnant women may be slightly lower than in healthy non-pregnant women. In view of current investigation, the mean platelet count in the pregnant women without malaria group was higher compared with pregnant women with malaria and non-pregnant women with malaria group respectively. The observed slightly lower mean platelet count in the pregnant women with malaria and non-pregnant women with malaria in this study points to the role of malaria infection as a prime factor contributing to the observed decrease in mean platelet count consenting with that of Verdy et al (416) who outlined slightly lower mean platelet counts in pregnant women compared to healthy non-pregnant women.

WBC are the key players of the body’s defense and WBC has been reported to be elevated during pregnancy (417). In view of current investigation, the WBC count in the pregnant women without malaria was lower as compared to the pregnant women with malaria and non-pregnant women with malaria group respectively. The finding of increase in WBC count in the malaria infected group from this study reinforces the argument of immunity building in the face of infection which is attained by a state of selective immune tolerance (418).

The decrease in haematological parameters such as Hb, RBC, PLT count in malaria in pregnancy group were not statistically significant which may be due to quality healthcare facilities available to pregnant women.

The decrease in HCT in pregnant women with malaria showed significant results. Limitations of the current investigation include lack
of prior medical history including anti-malarial treatment for the non-infected cases, which could potentially affect the explanation of the results. Furthermore, no further investigations were carried out to rule out other infection such as bacterial and viral that could lead to haematological alterations.
Graph 1 - Concentration of Haemoglobin in MIP and PWM

Fig: This Graph shows the concentration of Haemoglobin and Pregnancy without malaria. p-value calculated was <0.0001 by unpaired t-test using Graphpad Software
Graph 2 - Haemoglobin concentration in Malaria in pregnancy and women with malaria.

p-value calculated was < 0.0001 by unpaired t-test using Graphpad software.
Graph 3 - RBC count (mmol/L) in Malaria in pregnancy and Pregnancy without malaria

p-value calculated was <0.0001 by unpaired t-test using Graphpad Software
Graph 4 - RBC count in Malaria in pregnancy and Women without malaria
p-value calculated was <0.0001 by unpaired t-test using Graphpad software
Graph 5 - WBC count in Malaria in Pregnancy and Pregnancy without malaria

p-value calculated was <0.0001 by unpaired t-test using Graphpad software
Graph 6 - WBC count in Malaria in Pregnancy and women with malaria. The p-value calculated was <0.0001 by unpaired t-test using Graphpad software.
Cytokine level in both Non pregnant women and Pregnant women at different trimester

Graph 7 - Level of cytokine in both Non pregnant and pregnant women at different trimester.
CHAPTER 7
C - reactive protein profiling in clinical isolates of malaria in pregnancy and healthy pregnant subjects.

Malaria is pervasive in tropical regions, caused mainly by the protozoan parasites *P. falciparum* and *P. vivax*, accounts for 515 million clinical cases (419) and 1 to 3 million deaths per year (420) *P. vivax*, the most rampant parasite causing human malaria, accounts for about 130-435 million infections annually and is the prime cause of malaria in most of Asia and Latin America (421). Although *P. vivax* infection is commonly considered to be much more benign than *P. falciparum* infection, data do suggests notable mortality associated with *P. vivax* malaria in the pre antimalarial era (422) and death caused by *P. vivax* malaria has been alarmingly recognized over the past few years (421,423). CRP is an acute phase reactant and is one of the most widely used acute phase inflammatory proteins due to its early rise and rapid
dynamics. Assessment of CRP can be useful in understanding the aetiology of severe malaria (424). The protein is synthesized in the liver and is ordinarily present at a concentrations of less than 10mg/L in the blood. The level of CRP is alarmingly elevated during infectious or inflammatory diseases states, within the first 6 to 8 hours and extreme at levels of up to 350-400 mg/L after 48 hours (425-429).

CRP adhere to phosphocholine expressed on the surface of mutilated cells, as well as to polysaccharides and peptosaccharides present on bacteria, parasites and fungi (430). This binding stimulates the classical complement cascade of the immune system and regulates the activity of phagocytic cells, reinforcing the role played by CRP in the opsonization of infectious agents and dead or dying cells (425,430). After the resolution of inflammation or tissue destruction, CRP levels decline, fabricating its role as a valuable marker for tracking disease activity (426,429). Quantification of CRP is mostly done by using ELISA, immunoturbidimetry, or antibody-based nephelometric assays which are typically sensitive to concentrations of 5-20 mg/L. Nevertheless, in women CRP concentrations seem to be elevated in late pregnancy (429). Recently, some acute phase reactants have appeared as biomarkers in malaria infection in addition to chemokines and cytokines. Specifically, CRP and NO have been apprehended as an important inflammatory biomarker (431,432). In case of malaria, the secretion of CRP is promoted by pro-inflammatory cytokines that are secreted by host mononuclear cells and a strong positive coalitions have been noted between CRP levels and parasitaemia (433). In fact, CRP levels have been documented valuable in monitoring the severity of malaria among pregnant women and as a prognostic marker in due course of response to treatment (434).

Quantification of serum C-reactive protein is customarily used for the assessment of injury in the body tissue or for identifying inflammation in
the body. C reactive protein seem to play a pathogenic role in malaria infections. C-reactive protein adheres to infected erythrocytes and channelize its effective clearance. This augmentation of the immune system towards infected RBCs also results in various harmful expositions. Also, CRP trigger complement pathway and platelet activation resulting in various unpredictable effects. Thus, assaying CRP can be effective in interpreting the pathogenesis of severe malaria (424). Although CRP is classically reviewed as a key regulator of the innate immune system and a supreme mediator of the acute-phase response (435) it is also correlated to various chronic inflammatory processes, such as certain rheumatologic conditions (436). This study was precursory venture to support the prudent use of C reactive protein lab measurements, in the light of available scientific evidence and on the clinical experience of physicians. Accurate evaluation of severity of disease is the need to channelize proper therapy and avoid the complications (424) Specific data on the CRP levels in the pathology of malaria among pregnant women at this understudied district have not been documented in this regard. Therefore, the present study focusses to investigate the level of CRP in clinical isolates of MIP subject and HPW which could serve as a valuable prognostic marker in assessing disease severity.

Materials and methods:

Screening and enrollment:

A cross sectional study was conducted at Sadar Hospital, Hazaribag involving 68 malaria in pregnancy subjects who were confirmed to be infected, with either *P. vivax* or *P. falciparum* constituted the study population and 50 pregnant healthy women were enrolled as the control group. Out of 68 participants enrolled in the current investigation, 3 had *P. falciparum*, 59 had *P. vivax* infection and 6 had mixed infection. Inclusion
in the study protocol involved no history of hereditary diseases or known severe disease at the time of conceiving, participants who consented in the study protocol, no immediate illness due to other infectious disease or malaria during present pregnancy.

Exclusion from the study protocol involved refusal to give signed consent or unwilling for sampling, clinically suspected or identified cases of HIV and hepatitis B infection. About 3ml of peripheral venous blood was collected from the study participants for assaying C reactive protein.

**Ethical clearance:**

Written informed consent were taken from the study participants under protocol approved by the Institutional Ethics Committee of the Vinoba Bhave University, Hazaribag, Jharkhand and human ethical guidelines as contemplated in the guidelines of the Medical Ethics Committee, Ministry of Health, Govt. of India.

**Statistical Analysis:**

The data were collected, recorded and analysed statistically to ascertain the significance of means of various groups by using SPSS software version 21. Continuous variables were expressed as mean ±S.D. Post-hoc tukey’s test was performed for multiple comparisons between means of various group. A value of P˂0.05 was considered as statistically significant.

**Result:**

A significant correlation was found between CRP levels and parasitaemia. A statistically significant difference was observed between *P. falciparum* infected subjects (21±0.816 mg/l) and healthy pregnant women (11.52±0.499 mg/l) p<.0001. Significant difference have also been accorded between the CRP level of *P. vivax* infected pregnant subject
(22.084±1.093) and Healthy pregnant women (11.52±0.499) and mixed infection (23.5±4.03) and Healthy pregnant women (p<.0001).

**Discussions: -**

An interesting finding of the current investigation is an elevated level of CRP in malaria in pregnancy subjects as compared to its healthy pregnant counterparts (p<.0001). Strikingly, no significant difference between CRP levels in *P. falciparum* and *P. vivax* have been demonstrated (p=.101). Our finding corroborates with the study of Paul et al (424) who documented a significant peak value in CRP in malaria infected subjects compared to its healthy counterparts but predicated no significant difference between *P. falciparum* and *P. vivax* cases. Since, our study is a single time point study, it’s hard to infer precise assessment of the course of the disease.

Quantification of serum CRP concentrations can render a simple measurement of disease severity and could serve as a prognostic marker in malaria.
This Graph shows the concentration of C-reactive Protein in MIP-Pf and HPW. p-value calculated was <.0001 by unpaired t-test using Graphpad Software.

**Graph 8: - Concentration of C-reactive Protein in Malaria in pregnancy -Pf and Healthy pregnant women**
This Graph shows the concentration of C-reactive Protein in MIP-Mixed Infection and HPW.

p-value calculated was<0.0001 by unpaired t-test using Graphpad Software.

**Graph 9:** Concentration of C-reactive Protein in Malaria in pregnancy -mixed infection and Healthy pregnant women
Graph 10: Concentration of C-reactive Protein in Malaria in pregnancy -Pv and Healthy pregnant women

This Graph shows the concentration of C-reactive Protein in MIP-Pv and HPW p-value calculated was <0.0001 by unpaired t-test using Graphpad Software
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