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Role of IL-1β, IL-6 and TNF-α cytokines and TNF-α promoter variability in *Plasmodium vivax* infection during pregnancy in endemic population of Jharkhand, India

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ABSTRACT

Background: The combinatorial effects of *Plasmodium* infection, perturbation of inflammatory responses and the dichotomic role of TNF promoter polymorphism has potential clinical and physiological relevance during pregnancy.

Objective and Methods: This coordinated orchestration instigated us to investigate the circulating level of inflammatory cytokines (IL-1β, TNF-α and IL-6) employing ELISA in a stratified group of samples and the plausible genetic association of TNF-α −308 G/A using PCR-RFLP/sequencing during *Plasmodium vivax* infection in pregnancy.

Results: We observed significantly elevated concentrations of IL-1β were observed, followed by IL-6 and TNF-α in women with malaria (WWM) and in malaria in pregnancy (MIP). Further, elevated IL-1β, followed by TNF-α and IL-6 were detected in the non-infected pregnancy group. The differential dynamics of inflammatory cytokine concentration during each trimester of pregnancy with and without *P. vivax* infection were detected. For the first time, a high level of IL-6 was observed in the first trimester of MIP and high IL-1β in healthy pregnancies. In the second trimester, however, we observed a high level of IL-1β in the MIP group compared to a sustained high level of IL-1β in the healthy pregnancy group. In the third trimester, high IL-1β was sustained in the MIP group and healthy pregnancies acquired a high TNF-α level. The genotypic distribution for the TNF-α promoter −308 G/A position was observed to be nonsignificant and mildly associated during MIP (OR = 1.4) and in WWM (OR = 1.2). Moreover, based on genotypic distribution, we observed a well-correlated and significantly elevated TNF-α concentration in the mutant homozygote genotype (AA; $p = 0.001$) followed by heterozygotes (GA; $p = 0.0001$) and ancestral genotypes (GG; $p = 0.0001$) in both MIP and WWM subjects.

Conclusion: The observation of elevated IL-1β and IL-6 in MIP and TNF-α in WWM may be regarded as a prognostic inflammatory marker of infection and pregnancy. Most particularly, the TNF-α concentration and its polymorphic variability in the promoter region may indicate genetic susceptibility and mildly influence the risk for *P. vivax* infection during pregnancy and in women with malaria.

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1. Introduction

Pregnant women and their infants are more susceptible to common and preventable infectious diseases, including malaria, and most of this disease's mortality and morbidity is borne by children and pregnant women. With more than 300 million people suffering from malaria and over 50 million women exposed to the risk of malaria during pregnancy, and an annual infant fatality of 75,000–200,000, malaria is a serious public health concern across the globe (Desai et al., 2007). The dominant malaria parasites infecting humans, *Plasmodium falciparum* and *Plasmodium vivax*, contribute to pregnancy-associated malaria and cause various adverse pregnancy outcomes depending upon the clinical mediators and geographical locations (Dellicour et al., 2010). Both infections have unique features of pathogenesis, epidemiology and clinical course during pregnancy given that parasites can sequester in the placenta, reduce maternal hematocrit, and can cause massive inflammation most particularly at the maternal–foetal interface. Moreover, pregnant women have a lower resistance to malaria; in fact, they are four to 12 times more likely to have patent parasitaemia and are more susceptible to severe complications of the disease compared to other adults (Brabin, 1991).

Infection-induced physiological perturbation and immunological imbalance affects the overall inflammatory equilibrium of women's immune system necessary for a successful, full-term pregnancy. The counter balancing phenomena of anti- and pro-inflammatory cytokines is potent factor in the regulation of an effective immune response to malaria as well as role in supporting successful pregnancy via eliciting the coordinated orchestration of innate and adaptive immunity (Chene et al., 2014; Yasnot et al., 2013). More precisely, inflammatory cytokines have a dichotomic role in human immune responses to malaria disease and during pregnancy, although the balance and perturbation of inflammation alone and in conjunction with parasitic infection is less clearly defined and elucidated in humans. In pregnancy, uterine decidual macrophages (also called M1 macrophages) induce the development of an inflammatory phenotype that is characterized by elevated secretion of inflammatory cytokines (Nagamatsu and Schust, 2010). During a normal pregnancy, the Th1/Th2 activity balance is strongly shifted toward Th2 activity and it plays a potentially protective role in the foetal–maternal relationship (Souza et al., 2013; Sykes et al., 2012a). Inflammatory and infection processes alter the balance of Th1 and Th2 cytokines, causing a shift towards a Th1 predominance, which initiates and intensifies the cascade of inflammatory cytokine production involved in spontaneous abortion, preterm delivery, preeclampsia and labour (Chaouat et al., 2002). However, a delicate balance between pro-inflammatory and anti-inflammatory cytokines regulates inflammatory kinetics during pregnancy (Yilmaz et al., 2012). The regulated interplay between pro- and anti-inflammatory cytokines is a pivotal factor in determining malaria parasitaemia, birth outcome, clinical protection and rate of recovery (Riley, 1999), while overproduction contributes to adverse birth outcome, pregnancy-associated complications, immunopathology and disease progression (Umbers et al., 2011). Pro-inflammatory cytokines play a key role in orchestrating the complex events involved in inflammation and immunity (Peeters et al., 2001). Previously, it has been demonstrated that an elevated level of inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Friedland et al., 1993) was associated with disease severity in malaria during pregnancy (Brickley et al., 2015; Day et al., 1999). These cytokines are instrumental in influencing the killing and clearance of parasites from the placenta by enhancing the phagocytic activity of macrophages, generating reactive oxygen intermediates and L-arginine-derived nitric oxide, and stimulating the proliferation of T cells. Therefore, Th1-type responses are of parasitological importance. However, overproduction can threaten normal pregnancy, as the Th1 response is associated with maternal anaemia, spontaneous abortions and premature deliveries (Raghupathy, 1997).

The parasite's selective pressure on the human genome and the advent of molecular genetics has opened evidence-based approaches to numerous undisputed polymorphic loci that appear to contribute to malaria susceptibility or resistance and polymorphism-associated variability of malaria phenotypes (Weatherall and Clegg, 2002). Based on well-documented evidence, various TNF-α gene promoter loci have been assessed in disease-association studies (Gichohi-Wainaina et al., 2016; Olaniyan et al., 2016). Among the single nucleotide polymorphisms of TNF-α gene promoter at position −308, a G/A substitution has been shown to be associated with pregnancy (Stonek et al., 2007) and delivery outcome (Reddy et al., 2014) in various diseases, including in patients with severe malaria anaemia, asymptomatic malaria and cerebral malaria (Gounden et al., 2012). The established sensitivity of *Plasmodium* parasites towards inflammatory responses and the implication of a disturbed inflammatory network have attracted scientists to investigate inflammatory cytokines as a potential candidate in the diagnosis and monitoring of malaria-induced clinical complications and disorders during pregnancy.

Importantly, we have limited knowledge and understanding about the orchestration of inflammatory cytokines profile, TNF-α transition polymorphism in *Plasmodium* vivax-infected malaria patients during pregnancy and without pregnancy, globally and particularly from the perennially endemic transmission zone i.e. Hazaribag, Jharkhand, India. Considering the obstetric significance and immunomodulatory role of inflammatory responses during host-parasite interaction; we systematically investigated the circulating concentration of IL-1β, TNF-α and IL-6 in a clinically stratified group of subjects i.e. malaria during pregnancy, women with malaria without pregnancy, and in different trimesters of pregnancy with and without malaria infection attending an antenatal care unit (ANC) at Sadar Hospital, Hazaribag, Jharkhand, India as compared to region and age matched healthy women subjects. Additionally, we also investigated the transition polymorphism at −308 region of TNF promoter and its association with malaria in pregnancy; and to the best of our knowledge, there has been no published study examining the association and such comprehensive inflammatory profile on Indian isolates from the endemic region of Jharkhand. The rationale behind the present investigation was to explore the clinical spectrum of inflammatory interplay, host–parasite interaction, and to evaluate the potential of TNF-α, as prognostic immuno-genetic marker during infection and pregnancy from endemic population.

2. Methods

2.1. Study sites and population

A cross-sectional investigation was conducted in the ANC units of Sadar Hospital in Hazaribag district of Jharkhand, India (Fig. S1), a rural-cum-semi urban district, an eastern Indian state located in the tropical and forested zone with favourable geoclimatic and ecological conditions conducive to rich and distinctive flora and fauna biodiversity, considered to be a malaria-endemic area in the state of Jharkhand. Endemically, the study site has significance in view of low but perennial and asymptomatic transmission of malaria throughout the Hazaribag district with an average slide positivity rate (SPR) of 8.7% over the last 3 years (State Malaria Control Program Annual Report Ranchi, Jharkhand, Directorate of Health Services, 2010), with the burden of malaria mainly due to *P. vivax* in the majority of the indigenous population, a mix of tribals, Scheduled caste, Scheduled tribes and other casts; exceptionally, typical social stratification includes gender disparity (Sohail et al., 2015). The region is highly endemic for *vivax* malaria lacerated with women health issues including malaria in pregnancy (Sohail et al. 2015, Hamer et al., 2009) and to the best of our knowledge, such profile and clinical correlation has not been investigated before on Indian clinical isolates.

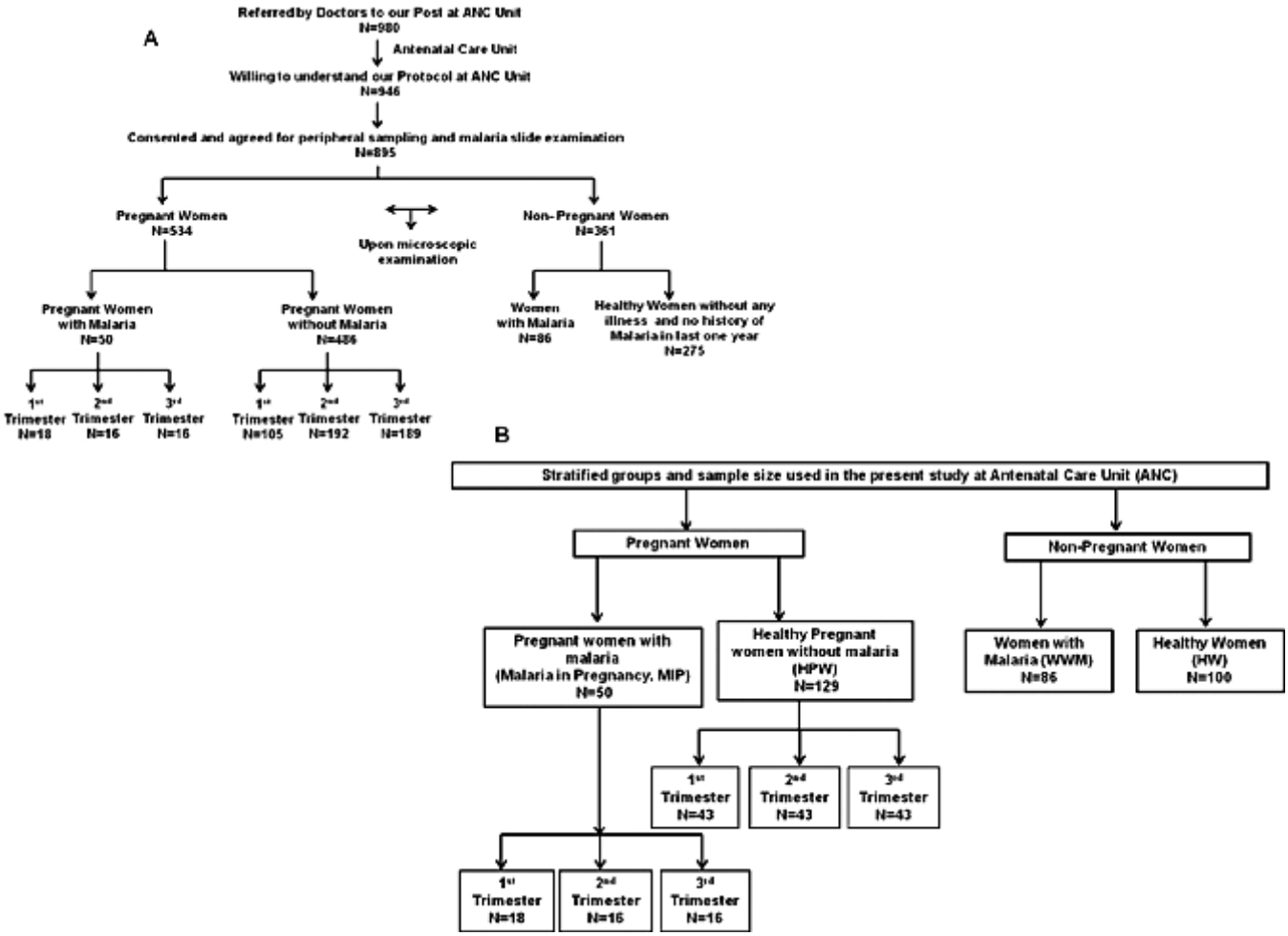

Fig. 1. Flow chart representing (A) the recruitment strategy at the antenatal care unit at Hazaribag's Sadar Hospital, Jharkhand, India. (B) Sampling strategy and subject stratification used in the investigation.

2.2. Screening and enrolment

Pregnant women aged ≥18 years presenting at the ANC for routine care were screened and enrolled, after providing informed consent to participate in the study, as per the schematic flow chart (Fig. 1A). Briefly, 980 were referred, out of which 946 understood the protocol and 895 consented to peripheral sampling: 534 were pregnant and 361 were non-pregnant. Upon microscopic examination, both the pregnant and non-pregnant groups were further categorized into pregnant women with malaria, pregnant women without malaria, women with malaria and healthy women. Both pregnant women with malaria and pregnant women without malaria were stratified into separate groups according to pregnancy trimesters. Each group and sub-group had specific enrolments and there was no overlap of sampling strategies throughout the investigation. The study subjects were recruited and enrolled from March 2014 to April 2015. In present study, the details of sample size in various stratified groups and sampling strategy at hospital was carried out as shown in Fig. 1B. Specifically, at the ANC, we analysed pregnant women (PW {malaria in pregnancy (MIP) + healthy pregnant women without malaria (HPW)}; $n = 179$), non-pregnant women (NPW {women with malaria (WWM) + healthy women (HW)}; $n = 186$), malaria in pregnancy (MIP; $n = 50$, further subdivided into trimesters; first trimester [1st TM; $n = 18$], second trimester [2nd TM; $n = 16$], and third trimester [3rd TM; $n = 16$]), healthy pregnant women without malaria (HPW; $n = 129$) further subdivided into trimesters; first trimester [1st TM; $n = 43$], second trimester [2nd TM; $n = 43$], and third trimester [3rd TM; $n = 43$]), women with malaria (WWM; $n = 86$) and healthy women (HW; $n = 100$) who were included in the analysis.

2.3. 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 prior anti-malarial treatment; and use of anti-malarial preventive measures. A complete physical examination including the determination of gestational age by palpation of uterine fundus height combined with information on the last menstrual period; axillary temperature and other vital signs were also measured by medical experts. Peripheral venous blood (3–5 ml) was collected from all the attendees for malaria blood film preparation, rapid diagnostic test (RDT) and haemoglobin determination apart from other biochemical and molecular investigations. Women diagnosed with positive RDT or anaemia were immediately referred to the hospital physician for treatment. The hospital staffs were informed to identify additional parasitaemic individuals through blood smears so that they could be appropriately treated.

2.4. Laboratory procedures

Thick and thin smears prepared from peripheral blood of the study subjects were Giemsa-stained and examined under a high-power microscope. The parasite density was evaluated by counting the number of

asexual forms of parasites in every 200 leukocytes, assuming a leukocyte count of 8000 leukocytes/μl of blood. The thin film was used to identify the *Plasmodium* species. All slides were cross-checked using stringent diagnostic criteria to diagnose *Plasmodium* infection by trained technical staff. The commercial (RDT kit) First Response Malaria pLDH/HR2 combo test kits (Premier Medical Corporation, Mumbai, India) were used as a screening tool for diagnosing malaria in pregnant women strictly according to the manufacturer's guidelines. Additionally, to rule out the possibility of submicroscopic infection; we have performed confirmatory molecular diagnostic PCR on all the cases and control samples included in our study.

2.5. Sample processing and assay for IL-1β, TNF-α and IL-6 content

Peripheral venous blood (3–5 ml) was collected as per the sampling strategy depicted in Fig. S2B before administration of antimalarial therapy, aseptically by dripping from the syringe without anticoagulant into sterile pro-clot activator coated tubes. Blood was allowed to coagulate in a refrigerator for 4–6 h at 4 °C before being processed by centrifugation. Sera were preserved in three to five aliquots at −20 °C until measurements were taken, and at −80 °C for long-term storage. The serum cytokine concentrations were determined by competitive ELISA micro-well plate-based assay using the commercially available BD OptEIA kit and reagents from BD Biosciences (San Diego, CA, USA). The standard samples, healthy controls and blanks were set up in duplicate for each plate; mean concentrations of the duplicate samples were used for calculation purposes. The assays were performed according to the manufacturer's instructions and optical densities measured using a microplate reader at a 450-nm wavelength.

3. DNA isolation

Blood samples were collected in anticoagulant vials or as dried filter spots from healthy subjects and all patients diagnosed with *P. vivax* infection in the ANC unit. Genomic DNA was extracted from peripheral blood using the standard phenol-chloroform extraction method or from dried blood spots on filter using the DNA isolation kit (QIAmp Blood Kit; Qiagen, Krefeld, Germany) according to the manufacturer's instructions.

3.1. Genotyping and analysis of the genetic polymorphism of the TNF-α −308 promoters

The genetic polymorphism at position −308 of the TNF-α promoter region was analysed by PCR amplification followed by restriction fragment length polymorphism (PCR-RFLP). Sequence-specific primers used for PCR amplification and analysis of the transition polymorphism G > A at the −308 position were carried out as per the detailed protocol described elsewhere by Sohail et al. (2008). For restriction digestion analysis, 10 μl of PCR product was digested with 2 U Bsp19 restriction enzyme (Clontech) at 37 °C for 6 h and the products were visualized using agarose gel electrophoresis as described elsewhere by Sohail et al. (2008).

3.2. Evaluation of polymorphism in TNF-α promoter position −308 G/A during malaria in pregnancy and malaria without pregnancy

To assess polymorphism in the TNF-α gene at the −308 position, a 107-base pair fragment containing the −308 position of the promoter region of the TNF-α gene was amplified on the blood samples collected from MIP subjects and HW at the ANC unit. Restriction digestion of the PCR product with the Bsp19 enzyme yielded either the undigested 107-base pair fragment (homozygous patients for allele TNF2, lacking the Bsp19 site) or three 102-, 87- and 20-base pair fragments (heterozygous patients) or two 87- and 20-base pair fragments (homozygous patients for the TNF1 allele), as visualized by running the digested product on non-reducing 3.5% agarose gel electrophoresis.

3.3. Ethics statement and subjects' consent

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

3.4. Data management and analysis

All clinical, demographic and anthropometric information was carefully checked for correctness and inconsistencies were resolved before analysis. Data were entered in MS-Excel and analysis was performed using SPSS v.16 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). For comparisons of groups, the Student *t*-test was employed and expressed as frequency counts (per cent). All biochemical data are presented as median (interquartile ranges) and compared using the *t*-test. Odd ratios (ORs) and 95% Confidence

Intervals (CI) for genotype were calculated with logistic regression to quantitatively assess the degree of association. The differences were considered statistically significant when the *p*-value obtained was < 0.05.

4. Results

Recruitment and enrolment took place from July 2014 to April 2015. The overall physical and clinical profile of the stratified subjects included in the study is depicted in Table 1 and their socio-demographic details are shown in Table S1.

Table 1
Physical and Clinical Characteristics of Stratified Subjects Evaluated at Antenatal Care Unit.

Parameters	PREGNANT GROUP			NON-PREGNANT GROUP		
	Pregnant women with malaria median (interquartile range)	Pregnant women without malaria median (interquartile range)	P^*	Women with malaria median (interquartile range)	Healthy women median (interquartile range)	P^*
Age (years)	22 (20–25)	23 (20–26)	0.001	30 (25–40)	23 (22–26)	0.0001
Weight (kg)	46 (45–51)	48 (44–53)	0.004	47 (40–51)	47 (44–51)	NS
BMI (kg/m^2)	24.7 (20.1–27.3)	23.4 (19.7–26.3)	0.002	21.3 (15.9–21.4)	20.1 (18.9–21.9)	0.004
Sys (mm/hg)	103 (96–114)	116 (111–127)	0.003	120 (107–130)	122 (107–134)	NS
Dys (mm/hg)	67 (59–77)	74 (72–81)	NS	80 (72–84)	77 (63–82)	NS
Temperature (°C)	38 (37.5–39)	37 (36.5–38.5)	0.001	38 (37–39.5)	36 (35–37)	0.001
Haemoglobin (g/dl)	9.2 (8.5–9.8)	9.6 (9–10.4)	0.001	10.4 (9.8–11.2)	11 (10.5–12)	0.002

Sys, systolic blood pressure; Dys, diastolic blood pressure.
* *t*-test.

4.1. Demographic and epidemiological description of antenatal clinic attendees

Most pregnant women attending the ANC were in the 18- to 38-year-old age range and had some level of formal education, as shown in Table S1. They had attended a median of one ANC visit (range, 0–9) during their current pregnancy and almost half of the attendees were primigravidae (51.7%). A positive diagnostic test for malaria during pregnancy was obtained in 19% of the total cohort (n = 263) at the ANC, as shown in Table S1. The mean density of parasitaemia in the women with positive blood smears was 332 asexual forms/μl (range, 187–489). Furthermore, malaria in women without pregnancy was observed to be 23.8% (86/361) in all screened groups of non-pregnant subjects at the ANC.

4.2. Inflammatory cytokine (IL-1β, TNF-α and IL-6) concentration in sera samples of stratified groups and healthy subjects at the antenatal care unit

Upon consent, we initially screened and recruited the pregnant subjects to investigate and explore the effect of pregnancy and infection as compared to healthy women. To understand the orchestration of inflammatory responses in pregnant and non-pregnant groups of subjects, we investigated the circulating concentration of inflammatory cytokines (IL-1β, TNF-α and IL-6) in stratified groups of subjects. Interestingly, we observed almost fourfold higher IL-1β and twofold higher IL-6 in the pregnant women than in the non-pregnant subjects, and the differences were statistically significant, whereas TNF-α was marginally higher in the pregnant group and the differences were not significant, as shown in Fig. 2A. Furthermore, we analysed the inflammatory responses in malaria infection in addition to pregnancy and evaluated the circulating concentration of the above-mentioned pro-inflammatory cytokines. A significantly higher IL-1β level was observed in both pregnant women and malaria-infected women than in healthy women, whereas a relatively higher IL-1β concentration was found in malaria-infected women compared to the pregnant group; these differences were significant, as shown in Fig. 2B. For TNF-α, we observed a significantly higher level in both the pregnant and malaria-infected groups as compared to healthy women. Surprisingly, no considerable differences in mean TNF-α concentration between the pregnant and malaria-infected groups was noted, and the differences were not significant, as shown in Fig. 2C. Significantly higher IL-6 concentrations were observed in both pregnant women and malaria-infected women than in healthy women. As expected, a higher IL-6 level was shown in the malaria-infected group relative to the pregnant group and the differences were highly significant, as shown in Fig. 2D. Overall evaluation of pro-inflammatory cytokines in broadly classified groups of subjects indicate that IL-1β and IL-6 were substantially higher in the malaria-infected group followed by the pregnant group.

4.3. Differential interplay among inflammatory responses and Plasmodium vivax infection with pregnancy and without pregnancy

In view of understanding the orchestrations of the inflammatory response, we analysed the inflammatory cytokines (IL-1β, TNF-α and IL-6) in *P. vivax* malaria during pregnancy (MIP), *P. vivax* malaria without pregnancy (WWM) and healthy pregnant women (HPW) as compared to healthy women (HW). The overall profile of inflammatory cytokines in the respective groups (Fig. 3A–C) and the comparative profile between malaria in pregnancy (P) and malaria in non-pregnancy (NP) are shown in Fig. 3D.

We observed a significantly higher IL-1β concentration in the WWM, MIP and HPW groups of subjects as compared to healthy

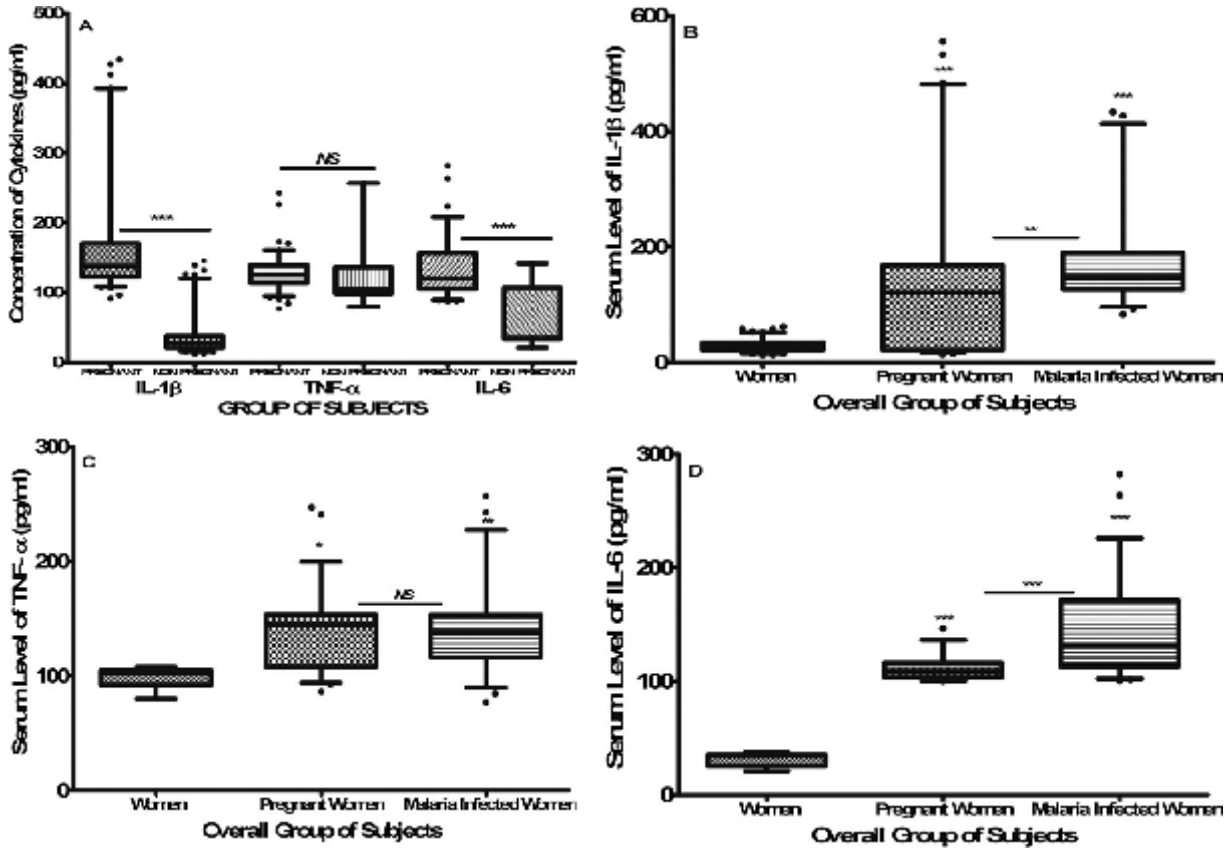

Fig. 2. Comparison of circulating level of cytokines. (A) Pregnant and non-pregnant groups of subjects; (B) IL1-β; (C) TNF-α and (D) IL-6 in malaria-infected women, pregnant women and healthy women, shown as median, interquartile range (box); 10th and 90th percentiles (whiskers) and statistical significance were deduced using t-test with GraphPad Prism 5.0 (* $p \leq 0.01$; ** $p \leq 0.001$; *** $p \leq 0.0001$).

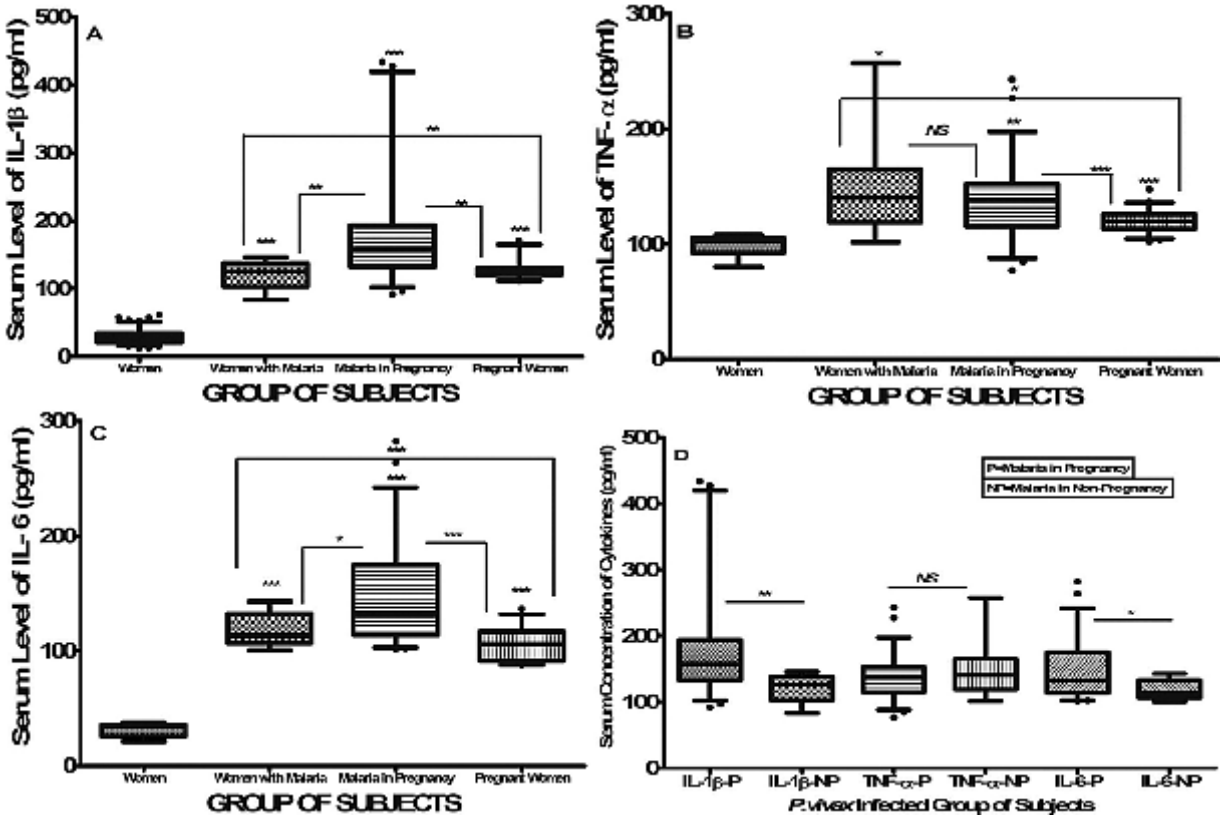

Fig. 3. Comparison of circulating level of cytokines. (A) IL-1β; (B) TNF-α and (C) IL-6 in stratified groups of malaria in pregnancy (MIP), women with malaria without pregnancy (WWM), healthy pregnant women (HPW) and healthy women (HW). (D) Comparative cytokine profile of *P. vivax*-infected women with pregnancy (P) and without pregnancy (NP) shown as median, interquartile range (box); 10th and 90th percentiles (whiskers) and statistical significance were deduced using the *t*-test with GraphPad Prism 5.0 (* $p \leq 0.01$; ** $p \leq 0.001$; *** $p \leq 0.0001$).

women. The highest concentration of IL-1β was found in the MIP group, followed by the WWM and HPW subjects. The relative differences in the cytokine concentration in the MIP vs HPW, MIP vs WWM, and WWM vs HPW groups were significant, as shown in Fig. 3A. In case of TNF-α, we found a high level in the WWM, MIP and HPW groups as compared to HW. However, the differences were significant in the WWM and MIP groups only. Interestingly, the highest concentration of TNF-α was observed in WWM, followed by the MIP and HPW groups. The relative differences in the cytokine concentration in the MIP vs HPW and WWM vs PW groups were significant only as shown in Fig. 3B. IL-6 was significantly higher in WWM, MIP and HPW as compared to the HW group. As anticipated, the concentration of IL-6 in the MIP group was highest, followed by WWM and HPW. The relative differences in the cytokine concentration in the MIP vs HPW, MIP vs WWM and WWM vs HPW groups were significant, as shown in Fig. 3C. To synchronize the responses of inflammatory cytokines and to differentiate between infection during pregnancy and non-pregnancy, these results indicate that IL-β and IL-6 are relatively significantly higher in MIP subjects, whereas TNF-α is relatively higher in infection with the non-pregnancy group of subjects, as shown in Fig. 3D.

4.4. Inflammatory cytokine profile according to pregnancy trimester with and without malaria infection

Apart from the physiological inflammation associated with progression of pregnancy in healthy women, the persistence of infectious diseases during pregnancy, particularly those caused by intracellular parasites such as *Plasmodium* spp., can potentially perturb the orchestration of the normal inflammatory response, and hence the cytokine profile, with possible relevance for the clinical severity and outcome of the disease. It should be noted that IL-1β was much higher in *P. vivax* infection during the third trimester of pregnancy as compared to the group without infection, whereas the IL-1β concentration was lower in infection during the second and third trimesters as compared to the group without infection. However, the differences in IL-1β concentration were statistically significant in all trimesters, as shown in Fig. 4A–C. Interestingly, we found that TNF-α was lower in *P. vivax* infection during all three trimesters as compared to the group without infection. However, the highest TNF-α levels in infection and without infection were observed during the second trimester of pregnancy and the differences in concentration were also only significant in the second trimester, as shown in Fig. 4B. We observed an elevated IL-6 concentration during all trimesters of pregnancy in malaria-infected women. However, a significantly different concentration was found only in the second trimester of pregnancy, as shown in Fig. 4B. More precisely, across the trimesters of pregnancy, we observed the dominance of high IL-6 followed by IL-1β in *P. vivax*-infected subjects, as shown in Fig. 4A–C. Additionally, this finding indicates that infection modulates the inflammatory responses during the first and second trimesters, although the overall dynamics of inflammatory cytokine concentrations were counter-regulated during pregnancy except for IL-6. Further towards the transition of the final trimester, the level of ambient inflammatory response was higher, as illustrated in Fig. 4A–C.

Taken together, these observations endorse the classical immunophysiological phenomena of normal pregnancy, which requires a finely tuned inflammatory environment during the entire gestation period and is of paramount biological significance for a successful pregnancy outcome.

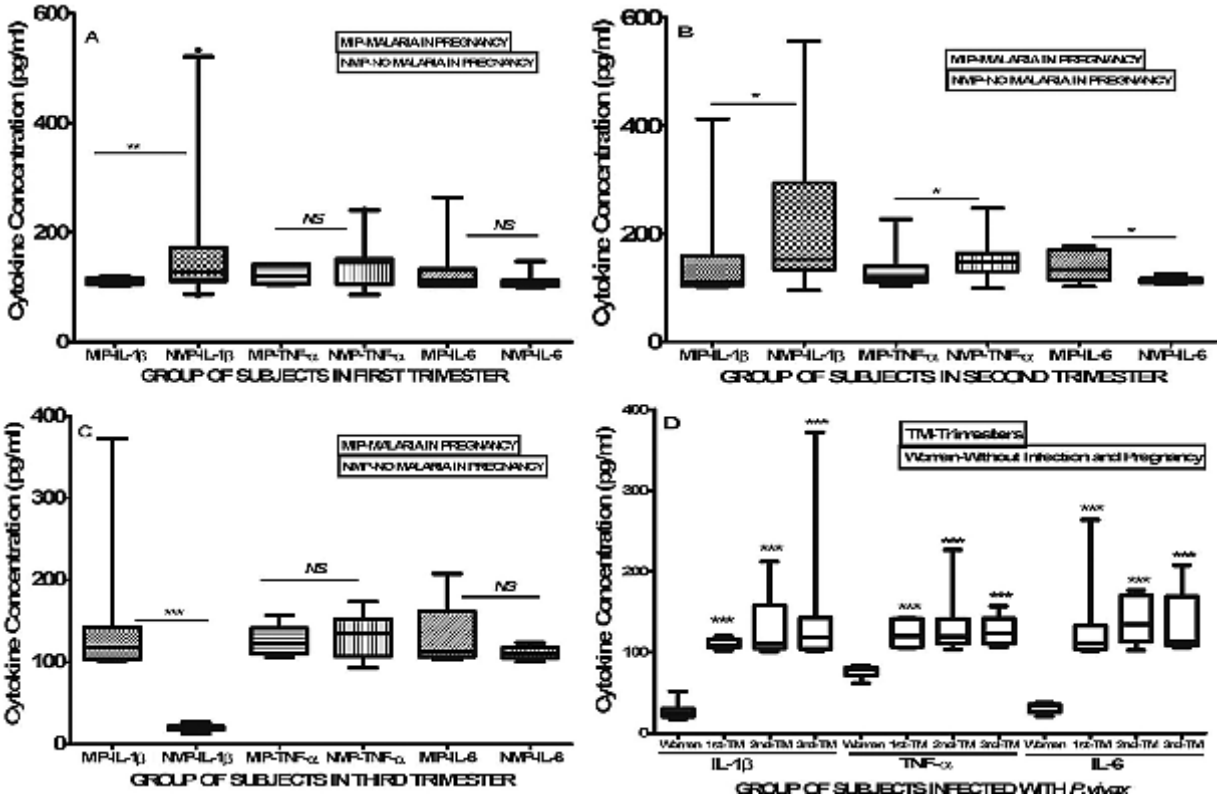

Fig. 4. Comparison of serum concentration of cytokines IL-1β, TNF-α and IL-6 between malaria in pregnancy (MIP) and no malaria in pregnancy (NMP) subjects during first (A), second (B) and third (C) trimester of pregnancy (D) Differential cytokines in infected trimesters, shown as median, interquartile range (box); 10th and 90th percentiles (whiskers) and statistical significance were deduced using *t*-test with GraphPad Prism 5.0 (* $p \leq 0.01$; ** $p \leq 0.001$; *** $p \leq 0.0001$).

4.5. Comparative evaluation of inflammatory cytokine profile in trimesters when pregnant and healthy women are infected with malaria

The cytokine profile of MIP subjects reflects a complex amalgamation of the physiological immune response generated due to pregnancy and the pathological immune response caused by persistent malaria. Both pregnancy and infection are potentially capable of altering the overall inflammatory cytokine profile, which can alter the clinical outcome of the disease and/or pregnancy. This prompted us to analyse the comparative effect of infection on inflammatory cytokines during the trimesters of pregnancy as compared to healthy pregnant women. Interestingly, we observed that IL-1β, TNF-α and IL-6 concentrations were substantially higher and differed significantly as compared to healthy women, as shown in Fig. 4D. This very useful observation indicates that differential and distinctive up-regulated concentrations of the inflammatory cytokines investigated herein may be significantly modulated by a combinatorial impact of infection and pregnancy (Fig. 4D), whereas pregnancy alone (Fig. 4A–C) and/or infection alone (Fig. 2B–D) has not shown such distinctive and differential up-regulation. Furthermore, this differential up-regulation of cytokine concentrations during *P. vivax* infection in pregnancy were nearly independent of pregnancy trimesters, as depicted in Fig. 4D.

4.6. Distribution of TNF-α genotype and allelic frequency in malaria during pregnancy and women with malaria consulting at an antenatal care unit

The genotype distribution for the −308 G/A position of TNF-α promoter in MIP and WWM subjects were compared with HW subjects, as shown in Table 2. The overall allele frequencies of −308A in MIP and in WWM subjects from the ANC unit were 40% and 37%, respectively, compared to 48% in HW. We observed no significant differences in the frequencies of −308AA promoter polymorphism of the TNF-α gene among *P. vivax*-infected malaria in the pregnancy and women with malaria groups as compared to healthy subjects (Table 2). However, the observed genetic frequency was non-significant in both the infected groups but mildly associated with and influencing the risk of malaria in pregnancy (OR 1.4; risk ratio 1.2) as well as in the WWM (OR 1.2; RR 1.1) group, as compared to the HW group. Furthermore, these genetic variability findings also have an impact on haematological perturbation, indicating moderate to severe anaemic complications during infection in pregnancy and WWM, as observed through significant lowering of the haemoglobin level in both the infected groups, as shown in Table 3.

4.7. Association of TNF-α polymorphism on TNF-α level in vivax malaria during pregnancy and in women with malaria

To understand the genetic regulation of the circulating concentration of TNF-α cytokine due to TNF-α promoter variability at position −308 and to explore its role during malaria in pregnancy, women with malaria without pregnancy and in healthy women, we evaluated the cytokine concentration based on genotypes. We observed significant and elevated circulating concentrations of TNF-α in the mutation specific to all stratified groups (HW, MIP and WWM) of subjects, as shown in Fig. 5A and B. More precisely, the highest mean concentration of TNF-α (194.33 pg/ml) was observed in the homozygous mutant genotype (AA) in malaria during pregnancy as compared to women with malaria (175.2 pg/ml), whereas the lowest mean concentration of TNF-α was found in the homozygous wild genotype (GG) in women with malaria (104.9 pg/ml.) as compared to malaria during pregnancy (136.23 pg/ml), as shown in Fig. 5A.

Furthermore, the circulating concentrations of TNF-α in mutant genotypes were markedly elevated and significantly associated with ancestral genotypes of malaria in pregnancy: in women with malaria the mutant genotypes were found to be almost 1.5-fold higher than the ancestral genotype, as shown in Fig. 5C and D. We compared the

Table 2
Genotypic distribution of TNF-α −308 G/A polymorphism among *P. vivax*-infected MIP and WWM subjects as compared to HW subjects.

Genotypes [a] Group of subjects	GG	GA	AA	χ^2	*P*	OR (95%CI)	Relative risk ratio
Malaria in pregnancy *n* = 50	14 (28%)	32 (64%)	4 (8%)	0.73	0.39	1.4 (0.6–3.4)	1.2
Healthy women *n* = 50	18 (36%)	16 (32%)	16 (32%)				
Women with malaria *n* = 50	16 (32%)	31 (62%)	3 (6%)	0.17	0.67	1.2 (0.5–2.7)	1.1

OR, odds ratio; CI, confidence interval.
[a] AA/AG vs. GG.

Table 3
Effect of TNF-α −308 polymorphism on haemoglobin content in malaria in pregnant and non-pregnant WWM subjects as compared to HW.

Based on mutation Group of subjects	Haemoglobin content median (interquartile range)				
Genotypes	GG	GA	*P**	AA	*P**
Malaria in pregnancy *n* = 50	9.1 (8.4–9.5)	8.5 (7.8–9.1)	0.02	7.5 (6.8–8.3)	0.002
Healthy women *n* = 50	10.7 (9.9–11.7)	10.5 (10–11.3)	*NS*	9.6 (8.7–10.7)	0.003
Women with malaria *n* = 50	9.3 (8.8–10.6)	9.2 (8.6–10.2)	*NS*	7.6 (7.4–8.2)	0.008

* Compared with wild genotype (*t*-test).

cytokine concentration in overall mutant and non-mutant genotypes of malaria in the pregnancy group, and interestingly the concentrations were found to be significantly elevated in the mutant group, as shown in Fig. 5E.

5. Discussion

Parasitic vulnerability during pregnancy, particularly to *Plasmodium*, has been regarded as one of the most heralded causes of obstetric and clinical complications due to the paradoxical link between underlying infectious agents and their ability to modulate maternal immunity. We investigated the interplay of inflammatory cytokines and *P. vivax* infection among female subjects with malaria in pregnancy, healthy pregnant women and non-pregnant women with malaria. The findings revealed the distinct and differential pattern of inflammatory cytokines, IL-1β, TNF-α and IL-6, in pregnant women as compared to healthy subjects. We found higher inflammatory cytokines in pregnant and malaria-infected women than in healthy non-pregnant women. These observations are in accordance with Girard et al. (Girard et al., 2014) and Sykes et al. (Sykes et al., 2012b). However, the patterns of inflammatory response differed from all the previously reported studies, suggesting region-specific immunological orchestration, linkage between maternal physiological and immune adaptation, ethnicity and

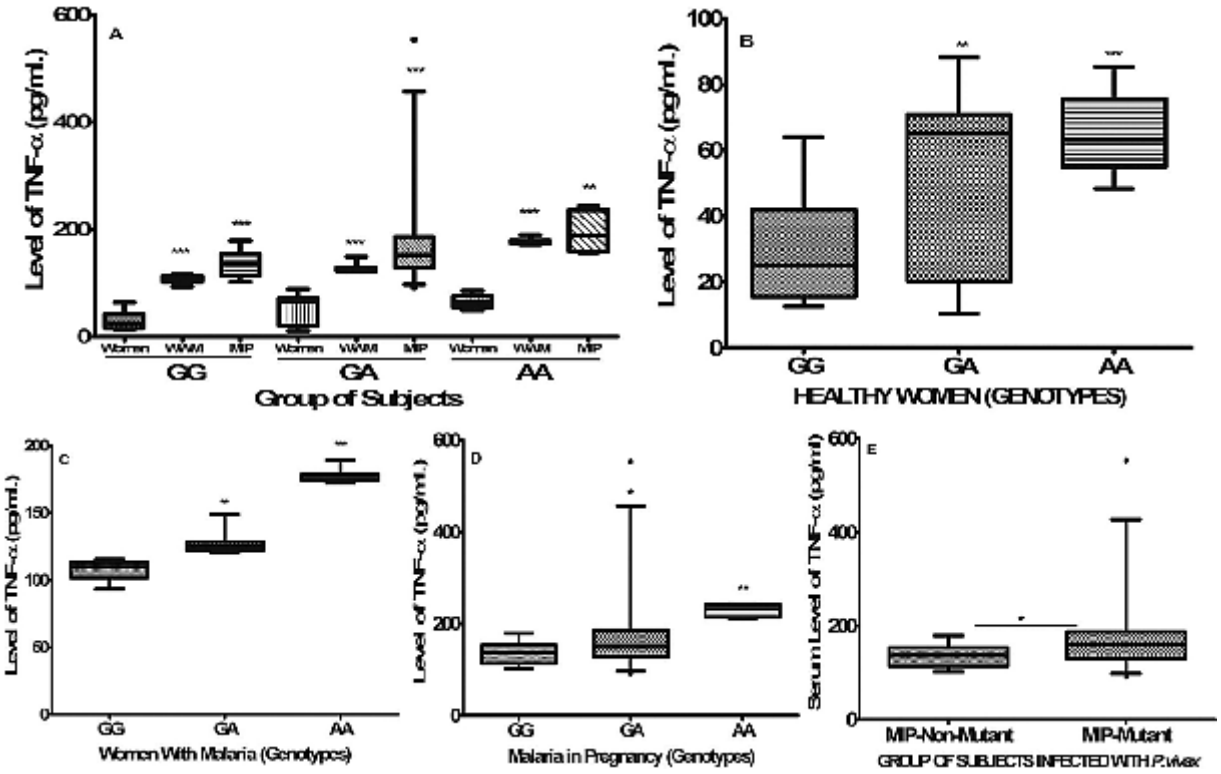

Fig. 5. Association of –308 TNF-α promoter polymorphism with circulating TNF-α concentration in malaria in pregnancy (MIP), non-pregnant women with malaria (WWM) and healthy women (HW). A) Comparison of circulating TNF-α concentration in stratified groups having wild-type (GG), heterozygous mutant (GA) or homozygous mutant (AA) genotype at the –308 position of the TNF-α promoter. B–D) Association of GG, GA and AA genotypes of –308 promoter position with circulating TNF-α level in HW (B), WWM (C) and MIP (D) subjects. E) Association of wild-type (GG, non-mutant) and mutant (GA or AA) genotype of –308 promoter position with TNF-α concentration in MIP subjects shown as median, interquartile range (box); 10th and 90th percentiles (whiskers) and statistical significance were deduced using *t*-test with GraphPad Prism 5.0 (* $p \leq 0.01$; ** $p \leq 0.001$; *** $p \leq 0.0001$).

systemic oxidative regulation (Mor et al., 2011).

Furthermore, these findings have biological significance and clinical relevance in regulating the normal gestational physiology, with increased pro-inflammatory cytokines such as IL-1β, IL-6 and TNF. They are believed to be important in facilitating uterine contraction, and intra-uterine growth retardation has been associated with labour as well as in establishing successful pregnancy (Moore et al., 1999; Osman et al., 2003). Th1-like and pro-inflammatory cytokines are also elevated in the blood of pregnant women compared to non-pregnant women (Sacks et al., 2003). High peripheral IL-1β concentration towards the end of pregnancy was previously observed at the foetal–maternal membranes or decidua at term delivery, whereas intra-amniotic infusion of the inflammatory cytokine IL-1β correlates with increased expression of IL-6, IL-8 and TNF-α, leading to increased uterine activity. They have been shown to play prominent roles in the cascade of inflammatory events associated with the onset of preterm labour (Romero et al., 2006), suggestive of critical regulation during pregnancy and the dichotomous role of these cytokines depending upon the immunological status of the host or the type and phase of the inflammatory process (Bamias et al., 2012).

P. falciparum malaria during pregnancy is a well-known cause of maternal and foetal morbidity, disease susceptibility, clinical severity and mortality as compared to *P. vivax*. (Nosten et al., 2004; ter Kuile and Rogerson, 2008). *P. vivax* is more common than *P. falciparum* in many parts of the tropics outside Africa (Desai et al., 2007), including the region of the present investigation (Sohail et al., 2015). Although *P. vivax* infection during pregnancy has been recognized for many years (Duffy and Fried, 2001), the impact of this infection during pregnancy has been assessed only recently. However, the deleterious effects of this infection alone and in association with other factors have yet to be characterized comprehensively. Intriguingly, all classical features of *P. falciparum* malaria during pregnancy such as asymptomatic susceptibility, relatively high prevalence of severe malaria anaemia, including clinical severity and symptoms, were inherently observed in *P. vivax*-mediated infection during pregnancy in Hazaribag, district of Jharkhand, India (Sohail et al. 2015). We investigated the comparative impact of host inflammatory cytokine responses in *P. vivax* infection during pregnancy, *P. vivax* infection without pregnancy and healthy pregnancy as compared to healthy women.

We observed higher concentrations of IL-1β, TNF-α and IL-6 in HPW than in HW (Fig. 3A–C), whereas TNF-α and IL-6 was higher in infected women as compared to HPW. These observations differed from the majority of previous investigations on this aspect, providing two new insights: host inflammatory cytokines were responding in a distinctively different fashion in pregnancy alone and in pregnancy with infection compared to infection in non-pregnant women. Secondly, we observed a higher concentration of IL-1β and IL-6 in malaria in pregnancy than in non-pregnancy. It can be inferred from this observation that *P. vivax* infection is the potent modulating factor for the host inflammatory cytokine responses in both cases, in infection alone and in association with pregnancy as compared to HW and HPW. These observations are in accordance with the observation made by Mendonca et al. (Mendonca et al., 2015). The plausible role of higher peripheral levels of inflammatory cytokines during *P. vivax* malaria may be sufficient to impair foetal growth and cause maternal anaemia, as hypothesized by Nosten et al. and Rogerson et al. (Nosten et al., 2004; Rogerson et al., 2007).

The immunological paradox of pregnancy is based on a fine balance of both immune tolerance and immune suppression via a trimester-specific shift of Th1 responses to a Th2 bias towards the end of the gestation period (Raghupathy et al., 2000). However, the majority of the gestational period up to successful delivery is predominated by an inflammatory environment (Saito et al., 2010). To elucidate this postulate, we evaluated and observed an increased trimester-specific concentration of inflammatory cytokines in maternal circulation of *P. vivax*-infected women with pregnancy as compared with healthy pregnant women. We observed marginal differences of inflammatory cytokines in both infected and healthy pregnancy groups in each trimester except for IL-1β concentration. IL-1β sustained significantly elevated levels in the third trimester of infected pregnancy as compared to their down-regulation in the third trimester of healthy pregnancy. However, elevated IL-1β levels were observed during the first and second trimesters of both infected and healthy pregnancy. This is in accordance with the observation made by Mor et al. (2011) and Requena et al. (2015), suggesting that strong inflammatory environments are required during implantation, placentation, and the first and early second trimesters of pregnancy (Mor et al., 2011). Additionally, IL-1β, together with TNF-α, stimulates the amnion, decidua and myometrium to express prostaglandins (Yilmaz et al., 2012) and promotes local progesterone metabolism (Roberson et al., 2012), which is necessary for maintaining pregnancy. The elevated inflammatory cytokine responses during the last trimester of pregnancy are a consequence of the physiological requirement for a renewed inflammatory environment to achieve the phenomena of parturition (Romero et al., 2006). Among inflammatory cytokines, IL-1β is the most influential inflammation mediator, which maintains pregnancy and regulates expression of various functional genes in myometrium smooth muscle cells and other cytokine secretions including TNF-α and IL-6 in epithelial cells and in the female reproductive tract (Romero et al., 2006), attributing to the elevated concentration of TNF-α and IL-6 in both infected and healthy pregnancy at the end of the third trimester (Mor et al., 2011). Furthermore, the wide variety of maternal genital and urinary tract infections (Fried and Duffy, 1998), postpartum anaemia, anaemia during pregnancy and primigravidas could be a possible reason for massive inflammatory responses (Fried et al., 1998; Sohail et al., 2015). The influence of host genetics on susceptibility and resistance to *Plasmodium* infection and their prospect as prognostic genetic markers in malaria during pregnancy prompted us to investigate the single nucleotide polymorphism in the inflammatory cytokine gene TNF-α −308 G/A promoter position to understand the role and association of this polymorphism with *P. vivax* infection during pregnancy.

Based on our observation of polymorphism data (Table 2); MIP (malaria in pregnancy) and WWM (women with malaria) group of subjects are mildly associated (OR = 1.4 and OR = 1.2, respectively) with lesser susceptibility to infection (Relative Risk (RR) 1.2 and 1.1, respectively) as compared to healthy subjects in the investigated population. This suggests the existence of one or more causal mutations within the TNF gene due to the confounding effect of both pregnancy and malaria infection, which may mildly influence the risk of the *vivax* malaria attack in the population investigated based on the odds and risk ratios observed (Table 2). The findings of the present study regarding genetic polymorphisms and the mild association with infection and/or the risk of malaria is substantially consolidated by the recent observation made by Nguyen et al. (2017) that among the four polymorphisms analysed, they found a nominal association between three of them (TNF-238, TNF-244, TNF-308) and the mild malaria attack in the Republic of Congo.

Our association of increased TNF-α levels in this case–control study are in accordance with the observations made by Andalas et al. (2015) and Liang et al. (2015). High serum concentrations of TNF in patients infected with *P. vivax* (Hajeer and Hutchinson, 2000; Karunaweera et al., 1992) may explain the general sickness of *P. vivax* infection observed even at low parasitaemia (Butcher et al., 1990). Kaijzel et al. (1998) observed that TNF among the monocyte-derived cytokines act in a paracrine fashion in the local environment to stimulate immune responses and also in an endocrine-like fashion on distant organs that participate in inflammatory responses in the diseased state. TNF-alpha appears to increase its own production by a positive feedback mechanism (Amiot et al., 1997) with a significant effect on the outcome of responses. However, there is no exact biological rationale so far to provide support for this effect. Elevated TNF concentrations resulting from polymorphism contributes to the systemic inflammatory

environment, although this is clinically dichotomic and elusive in view of its role in pathogenesis of symptoms associated with malaria infection (Bamias et al., 2012). It also regulates other physiological and endocrine functions during pregnancy and most importantly a regulated level of TNF-α is essential for enhancing phagocytic activity and controlling parasite density (Clark et al., 1990).

The frequency distribution of TNF-α − 308A/A in *P. vivax*-infected groups was observed to be lower (8% in MIP, 6% in WWM) than in healthy subjects (32%) in the present study, but not significantly different in the population studied (Table 2) and in accordance with the observations made by Sohail et al. (2008), Furini et al. (2016) and Afridi et al. (2012) in terms of lower prevalence of mutant genotypes in infection as well as non-association and non-significance in the respective populations investigated.

Furthermore, our observation of the high frequency of the mutant allele is in accordance with the observations made by Reddy et al. (2014) (35%) and Gounden et al. (2012) (34%) in a South African population. The reasons for observations of genetic disparities and the varied degree of association of TNF-308 with disease susceptibility can be attributed to various factors: (1) mutations profoundly influence several malarial phenotypes in different ethnicity and etiological conditions Nguyen et al. (2017) and (2) the TNF homozygous mutant at −308 is a stronger transcriptional activator Wilson et al. (1997) and very close to the binding site of the helix-turn-helix transcription factor OCT-1, which alters gene expression Knight et al. (1999). Although it has not been confirmed in vivo that TNF expression per se is controlled by its promoter variants, polymorphic chromosomal locations with extensive linkage disequilibrium and cis-acting transcriptional regulation were notable and probable causes of these findings. Moreover, the geographical differences in the population studied can also contribute to genetic association and disease outcome Ramasawmy et al. (2007), and given this community-based investigation, the small sample size is always a potential influencing and limiting factor that cannot be ruled out for these observations. Additionally, the biological plausibility of the significance of the observation can be attributed to the fact that cytokines may stimulate beneficial antiparasitic immunological responses in malaria by inducing an acute-phase response, inhibition of parasite growth, clearance of vascular parasites, debris and neutrophil-mediated increased parasite destruction (Hugosson et al., 2004). Regardless of the exact functional relevance, existing evidence and based on our findings, we hypothesize that the risk allele −308A in the TNF-α gene promoter does not significantly influence the risk of *vivax* malaria in pregnancy and in women with malaria, but it is associated with a circulating concentration of TNF-α in *P. vivax* infection during pregnancy and in women with malaria. The lower frequency of −308A implies that the mutant allele could provide protection from *P. vivax* infection and disease progression in the population investigated.

Precisely, our findings of inflammatory cytokines and TNF-α promoter transition polymorphism elucidating orchestration during infection, inflammation and pregnancy provide new insights into the immunological shifts, perturbation of the Th1/Th2 balance, skewing of protective maternal immunity and host genetic and host–parasite interacting factors over the course of pregnancy. The most intriguing and thought-provoking result of this study is the consolidation and sustenance of the pro-inflammatory shift of cytokines initiating from the first trimester of pregnancy to the end of pregnancy, i.e. the late third trimester in case of *P. vivax* infection during pregnancy. Our data also reveal that infection-mediated modulation of differential inflammatory responses not only depends on *P. vivax* infection, but also on the different stages of gestation, and pregnancy is susceptible to infection. We also acknowledge that this study has other limitations to be elucidated in future studies, such as screening for placental malaria, the placental cytokine profile and asymptomatic infection.

In conclusion, this investigation showed a non-significant lower frequency of the risk allele in infection and pregnancy, a moderate genetic association of the TNF-α promoter position −308 mutant homozygosity and a milder risk of *P. vivax* infection during pregnancy and in women with malaria, indicating the protective genetic orchestration in the malaria-endemic population of Hazaribag, Jharkhand, India. Furthermore, accumulative data suggest the potential cross-linking interplay of ethnic immunity, infection, disease transmission dynamics and polymorphism through a complex interplay of regulated pro-inflammatory cytokines. Both IL-1β and IL-6 are distinctively elevated in case of malaria in pregnancy, followed by pregnancy alone as compared to quite low concentration of both IL-1β and IL-6 in healthy women. The differences are over 10–12 fold between case and control and this could be distinctive and predictive marker for malaria infection based on investigated observation. However, possibility of this differential observation due to other infection is ruled out, as all the investigated pregnant subjects were also underwent clinical test (TORCH panel and HIV), exclusively examined by gynaecologist and have taken appropriate care in recruitment, sampling and clinical investigation. So far genetic susceptibility and association is concerned; we will suggest the evaluation of TNF-α polymorphism study, as it up regulates the circulating concentration of TNF-α as well as genotypic distribution helps in analyzing the association and relative risk factor in the investigated population. Thus, differential dynamics of circulating concentrations of cytokines may serve as an immuno-diagnostic marker and as prospective predictors of malaria during pregnancy, inflammatory perturbation in pregnancy and in gestational complications during certain trimesters of pregnancy. Rapid and early sub-clinical diagnosis based on circulating biomarkers will add to the existing and conventional diagnostic strategies for MIP. This will facilitate curbing the menace of morbidity and mortality due to malaria in pregnancy in general and particularly in endemic regions across the globe, including Jharkhand, India.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2018.03.019.

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