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Research Paper

iNOS polymorphism modulates iNOS/NO expression via impaired antioxidant and ROS content in *P. vivax* and *P. falciparum* infection

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ABSTRACT

Nitric oxide (NO) has dicotomic influence on modulating host-parasite interplay, synchronizing physiological orchestrations and diagnostic potential; instigated us to investigate the plausible association and genetic regulation among NO level, components of oxidative stress, iNOS polymorphisms and risk of malaria. Here, we experimentally elucidate that iNOS promoter polymorphisms are associated with risk of malaria; employing mutation specific genotyping, functional interplay using western blot and RT-PCR, quantitative estimation of NO, total antioxidant content (TAC) and reactive oxygen species (ROS).

Genotyping revealed significantly associated risk of *P. vivax* (adjusted OR = 1.92 and 1.72) and *P. falciparum* (adjusted OR = 1.68 and 1.75) infection with SNP at iNOS-954G/C and iNOS-1173C/T positions, respectively; though *vivax* showed higher risk of infection. Intriguingly, mutation and infection specific differential upregulation of iNOS expression/NO level was observed and found to be significantly associated with mutant genotypes. Moreover, *P. vivax* showed pronounced iNOS protein (2.4 fold) and mRNA (2.5 fold) expression relative to healthy subjects. Furthermore, TAC and ROS were significantly decreased in infection; and differentially decreased in mutant genotypes.

Our findings endorse polymorphic regulation of iNOS expression, altered oxidant-antioxidant components and evidences of risk association as the hallmark of malaria pathogenesis. iNOS/NO may serve as potential diagnostic marker in assessing clinical malaria.

1. Introduction

Malaria is a serious public health concern across the globe with a preferential dominance in tropical regions, including India. Despite of worldwide initiatives and efforts for prevention, prompt diagnosis, curative measures and possible eradication strategies; the global burden of malaria as a life threatening disease continues to worsen globally with a deplorable impact on human health and corresponding impediment to economic development. According to the recent estimate of WHO, 214 million cases of malaria occurred globally in 2015 and 43800 deaths; about 88% of the cases were from African region and 10% were from South-East Asia Region (SEAR) countries (WHO, World malaria report- 2015). India contributes to 70% of malaria cases and 32 per cent of malaria deaths among SEAR countries. Approximately 569 million people reside in high transmission areas in India, i.e. defined as more than one case per 1000 population (WHO-2013 [1]). *Falciparum* malaria accounts for approximately 247 million cases and one million deaths annually, particularly in sub-Saharan Africa [2] while outside the African continents, *Plasmodium vivax* is responsible for more than 50% of all malaria cases [3]. Outside of Africa, *Plasmodium falciparum* and *P. vivax* almost invariably coexist and are often equally prevalent [4], yet the public health importance of *P. vivax* is frequently overlooked [5]. *Plasmodium vivax* threatens almost 40% of the world's population, causing an estimated 72–390 million clinical infections each year [3,6].

Malaria is not uniformly distributed in India; ranging from sectarian low to high transmission zones with a prevalence of perennial transmission in malaria endemic regions. However, transmission intensities

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and incidence rates differ by seasonal changes, punctuated by a risk of epidemic every three to five years period. Higher and stable transmission were observed in geographically conducive i.e. forested and hilly areas with a sizable tribal settlements in the state of Andhra Pradesh, Jharkhand, Gujarat, Madhya Pradesh, Chhattisgarh, Orissa and Rajasthan. Jharkhand is an understudied and tribal prevalent region with perennial malaria transmission zone where malaria is rampant and causing 20×10^3 annual malaria deaths, second to Orissa in India as per the latest observation published by Dhingra et al. [7], which reflects the importance of the area and the necessity of undertaking extensive investigation in terms of malarial pathology is concerned. The morbidity in Jharkhand ranges from 1.5 to 2.3 Lakh cases annually, whereas, the mortality ranges from 16 to 35 cases annually over the last three years as per the Directorate of National Vector Born Disease Control Programme, India and Ministry of Health and Family Welfare, Govt. of India.

In view of the augmented pathology, poorly elucidated disease progression and underlying mechanisms, intriguing clinical variability, selective drug pressure and their discriminate use resulting into varied level of resistance, long awaited efficient therapeutic interventions and distant insights for effective and protective vaccine; all accumulated factors continue to perplex the situation for parasitologist over the past century but the reason and mechanisms of which remain enigmatic. This very situation demands exploration of alternative domains such as descriptive genetic epidemiology; which may open new vistas in understanding the role of genetic factors involved in resistance/susceptibility to diseases. Genetic factors play a key role in disease diagnosis, susceptibility and progression, and have translational significance for developing strategies to control the disease. Malaria parasites, as one of the oldest known parasite infecting humans, have had a long evolutionary host-parasite association. Among the various associating factors, identifying polymorphic variability in the parasite and the host candidate genes influencing disease risk and severity to plasmodium infection, is of paramount significance. Considering the magnitude of public health concern, we decided to select a gene from the bigger partner of association i.e. host gene and among them, nitric oxide (NO) was chosen as a potential candidate gene in view of its documented role in host defense machinery against infectious invasion by a variety of organisms [8,9]. A number of studies, both in-vitro and in-vivo especially from laboratory models of various protozoan infections including plasmodium, implicated nitric oxide as an integral component of the host armament against invading parasites and infectious agents. The underlying mechanism by which nitric oxide mediates defensive orchestration is either through direct parasite killing or by limiting parasite growth [10–12], though the working efficiency depends upon the various factors like site of action, timing and amount of its production and biological milieu in which it is released [10]. However, precise clinical relevance and role of nitric oxide in malarial etiology is dicotomic, as some investigators have associated NO with severity of malaria, particularly cerebral malaria [13–17], whereas, others opine that nitric oxide has a protective role [18–23]. Upon the triggering of immunologic or inflammatory stimulus, NO is constitutively produced from monocytes/macrophages by the enzymatic action of nitric oxide synthase. The enzyme catalyzing the production of nitric oxide is differentiated on the basis of their origin i.e. endothelial (eNOS or NOS3), neuronal (nNOS or NOS1) and the most abundant in concentration, the inducible nitric oxide synthase (iNOS or NOS2) [24]. The most sustained and highest production of nitric oxide is induced by iNOS [25] and contributes in intrahepatic killing of parasites in response to IFN-γ, TNF-α, IL-1β and IL-6 secreted by antigen-specific T cells and NK cells [26,27]. Production of nitric oxide is regulated through the enzymatic induction of NOS gene and it has been reported that NOS gene as host genetic factor do contribute to the variation in the frequency and intensity of clinical episode of malaria [28,29] and other infection [30]. Several NOS2 promoter polymorphisms have been studied in context of malaria pathology and severity. However, single nucleotide polymorphisms (SNPs) in the promoter region of the encoding gene at −954G/C and −1173C/T have been shown to increase NO synthesis [31,32]. Initially these polymorphism were observed to be associated with African populations [33] but recently similar observations were reported in studies extended to other malaria-endemic regions, such as Tanzania [34], Americans of African origin [34], Southeast Asia [35,36], white Americans [34] and Germans [20]. Additionally, interesting but ambiguous reports regarding the role of NO in malaria emerged from two recent studies; Kun *et al.* [20] demonstrated an association between polymorphism in the promoter region of the iNOS gene and protection from severe malaria in a Gabonese population, whereas, observations made by Burgner et al. [13] on Gambian population were somewhat different. Thus, the role of iNOS polymorphisms may vary with endemic regions across the globe as does the manifestation of malaria. Due to the confounding evidences [34–36] on NOS polymorphisms, particularly iNOS, and its functional importance, a number of studies have been carried out to investigate the role of these polymorphisms in disease susceptibility and protection, particularly in malaria and other infectious diseases. Although iNOS is an important gene involved in the regulation of gene expression, secretion of NO and host defence mechanism against various infectious and parasitic organisms, a systematic study of common genetic variations in this gene, its association with malaria pathology and impact on nitric oxide content has not been reported from malaria endemic population of Jharkhand, India. Thus, our objective of the investigation was to analyze the differential content of nitric oxide, other associated biochemical markers in order to evaluate the role and plausible association between iNOS-954G/C and −1173C/T transition polymorphisms and risk of malaria in the investigated population. Additionally, the other objective was to understand the translational impact of these polymorphisms on the expression of iNOS/NO pathway and components of oxidative metabolism on patient's actual responses upon plasmodium infection. In this study, we investigated whether these polymorphisms in the promoter region of the iNOS gene are influencing the risk of plasmodium infection, associated with circulating level of nitric oxide, and either corroborating antioxidant and ROS content or modulating the expression of iNOS/NO level in a species specific infection as compared to healthy subjects. Among the findings of these studies provides prominent insights that transition polymorphisms in iNOS promoter showed association of genetic susceptibility to the risk of malaria and critically regulate the expression of iNOS/NO level in species specific infection of plasmodium, in addition to promising rationale for diagnostic potential in human malaria. However, there has been no published study evaluating the orchestration among the biochemical components, impact of plasmodium infection and polymorphic regulation of iNOS expression in clinical isolates from malaria endemic zone of India.

2. Material and methods

2.1. Study sites and population

A prospective, cross-sectional investigation was conducted in the general OPD of Sadar hospital in Hazaribag; a tribal prevalent area representing endemic with stable transmission of malaria district of Jharkhand, India. The detailed description about the importance of study site, potential necessity of investigation, overview and socio-demographic status of the investigated population has been described elsewhere by Sohail et al. [37].

2.2. Patients and demographic information

A total of 320 malaria patients and 210 healthy persons (those without any evidence of parasites on microscopic examination) of either sex, who attended to the local Malaria Counter at Sadar Hospital, Hazaribag, Jharkhand and were referred by general physicians of the

Table 1
Demographic and clinical characteristics of the malarial patients and healthy subjects.

Parameters	Malaria Patients Mean ± SE	Healthy Subjects Mean ± SE	*P*
Number of Male/Female	157/163	104/106	
Age (Year)	26.7 ± 1.03	32.9 ± 1.38	0.0006
Temperature (°F)	100.1 ± 0.37	98.2 ± 0.05	0.0001
Weight (Kg.)	42.6 ± 0.99	57.6 ± 1.37	0.0001
Height (Feet)	5.0 ± 0.04	5.2 ± 0.03	0.0006
BMI (Kg/m^2)	18.1 ± 0.36	22.4 ± 0.4	0.0001
Systolic (mmHg)	113 ± 1.58	119 ± 1.91	0.001
Diastolic (mmHg)	74 ± 1.1	73 ± 1.42	*NS*

hospital and other parts of the districts between August 2012 to December 2014, were included in this study. Inclusion and classification of each case were based on the symptoms, physical signs and laboratory findings of malaria at the onset of disease. On the basis of the clinical investigation, parasite slide examination and measurement of axillary body temperature at attendance, patients were defined by (i) fever (axillary temperature $\geq 37.5\ ^0F$, or history of fever in the previous 24 h) with or without other symptoms of malaria (headache, body aches and malaise), (ii) the presence of any asexual *P. falciparum* density, (iii) no symptoms or signs of severe malaria or/and cerebral malaria, and (iv) no other etiology of fever discernible on clinical examination. Stringent diagnostic criteria were used to diagnose malaria infection with our trained technical staff. The healthy group included 210 adult relatives and attendants of the patients from same geographical area and similar ethnicity having no history of malaria in the last two years. Data collected included basic demographic information, pattern and onset of infection and use of medications. The subject characteristics and clinical data are presented in Table 1.

2.3. Sample collection and clinical investigation of infected subject

Peripheral venous blood (3–5 ml) was collected from all the patients before administration of anti-malarial therapy, aseptically by dripping from the syringe into a sterile pro-clot activator coated tubes for assessment of serum nitric oxide and in anticoagulant (EDTA) coated tube for DNA isolation. 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 immediately stored at −20 °C, and maintained at −80 °C until measurements were performed. Blood specimens were collected of all age groups during different transmission periods of the year from positive cases of malaria, who had undergone clinical investigation and confirmed on the basis of clinical symptoms. *P. vivax* or *P. falciparum* infection was confirmed by thick and thin-smear blood films using standard JSB staining techniques and light microscopy [38]. All the included patients were found to be infected with either *P. vivax* or *P. falciparum* but not both. However, commercial (RDT kit) First Response Malaria pLDH/HR2 combo test kits (Premier Medical Corporation, Mumbai, India) were also used as per the manufacturer's guideline and PCR was used as a screening and verification tool for diagnosing malaria in addition to microscopy.

2.4. Measurement of nitric oxide content

Serum samples were thawed at room temperature and centrifuged at 14,000 rpm for 10 min at 4 °C before processing of samples for deproteination required for nitric oxide estimation. Briefly, deproteination was carried out by mixing 150 µl of serum with 8 µl of $ZnSO_4$. Subsequently, 8 µl of NaOH was added, vortexed and centrifuged at 14,000 rpm for 10 min at 4 °C. 100 µl of clear supernatant was used for estimating the nitric oxide. Circulating levels of nitric oxide were quantified in duplicate by a colorimetric method with a linear detection range of 0.6–200 µM using a QuantiChrom™ Nitric Oxide Assay Kit (Bioassay Systems, Hayward, CA) according to manufacturer's instructions and optical densities measured using a microplate reader set to 540 nm wavelength. The arithmetic mean of the duplicate samples was considered for analysis.

2.5. Measurement of total antioxidant capacity (TAC) and reactive oxygen species (ROS)

Total Antioxidant Capacity (TAC) were quantified by a colorimetric method with a linear detection range of 1.5–1000 µM using a QuantiChrom™ Antioxidant Assay Kit (Bioassay Systems, Hayward, CA) according to manufacturer's instructions and optical densities measured using a microplate reader set to 570 nm wavelength. Briefly, in this method Cu^{2+} is reduced by antioxidant to Cu^{+} and resulting Cu^{+} specifically forms a coloured complex with a dye reagent. The colour intensity at 570 nm is proportional to TAC in the sample.

Intracellular ROS levels were measured in malaria infected (*P. falciparum* and *P. vivax*) and healthy subject's cells. PBMC were cultured for 24 h and then harvested, re-suspended in phosphate buffered saline (PBS) and the cell number was counted in a neubauer chamber. Further cells were incubated with H_2DCFDA (20 µM) for 30 min at 37 °C. Fluorescence was measured spectrofluorometrically at 530 nm using an excitation wavelength of 507 nm. For all measurements, basal fluorescence was subtracted.

2.6. Genotyping of iNOS-954G/C and iNOS-1173C/T transition polymorphism

The iNOS-954G→C polymorphism was determined using the PCR-RFLP method, whereas the iNOS-1173 C→T polymorphism was determined using mutation specific (MS)-PCR. PCR was used to amplify the fragments that contained the selected iNOS polymorphic sites as described by Levesque et al. [34] and Kun et al. [20,34], respectively. DNA was extracted from blood spots dried on filter paper using a DNA isolation kit (QIAmp Blood Kit: Qiagen, Krefeld, Germany) according to the manufacturer's instructions. The iNOS-954G→C polymorphism was determined using the PCR-RFLP method, whereas the iNOS-1173 C→T polymorphism was determined using mutation specific (MS)-PCR. PCR was used to amplify the fragments that contained the selected iNOS polymorphic sites. The primers of iNOS-954G→C were as follows: 5'-CATATGTATGGGAATACTGTATTTCAG-3' (forward) and 5'- TCTGAAC TAGTCACTTGAGG-3' (reverse), as previously reported by Levesque et al. [34]. The primers of iNOS-1173 C→T were as follows: 5'-GACA AGAAGGAAATGAGTGGACACAGGTAGCAAAGTGTTGAGAC-3'(MS-P2F),5'-GCATTTTTCCATCATAAAAGTAA-3'(MS-P3R) and5'- GTGGTA GCAAATGTTGGAAT-3'(MS-P4F), as previously reported by Kun et al. [20]. In order to detect the variants of −954G/C and −1173C/T of iNOS promoter, the isolated DNA (100 ng) were amplified as described previously [20,34].

2.7. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from both malaria infected and healthy subjects by diluting blood with PBS

on Ficoll-Paque Plus (GE Healthcare Life Sciences, India) and centrifuged at 400*g* for 30 min at room temperature following manufacturer's standard guideline. PBMCs were carefully aspirated from Ficoll-Plasma interface and washed twice with PBS at 300*g* at 4 °C and resuspended in RPMI 1640 and acclimatized for overnight growth in culture. Further, before subsequent use in RNA isolation and immunoblotting, cells were assessed by trypan blue exclusion test and over 90% viability was observed.

2.8. Evaluation of NOS protein in malaria patients by Western blot analysis

For evaluating the induction of iNOS protein expression from *P. vivax*, *P. falciparum* and healthy subjects; PBMCs were thoroughly washed twice with ice-cold PBS and resuspended in ice cold RIPA buffer for lysis with 1% Triton X100% and 0.1% SDS following the methods as previously described [11] and visualised by the procedure of staining using Colloidal Coomassie Blue G-250. Protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins (30 micrograms) were separated by SDS-PAGE, transferred to PVDF membrane sheets (Millipore, Billerica, MA, USA) and probed with the anti-iNOS (BD Biosciences; rabbit polyclonal, diluted 1:1000) and HRP-conjugated anti-mouse for iNOS secondary antibody (at 1:2000 in 1% skimmed milk TBST), and detection was done by enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ) Western blotting detection reagents to visualize immunoreactive bands. Immunoreactive bands were digitally scanned and analyzed with NIH ImageJ for quantification by densitometry as protein expression relative to that of healthy subjects. The human β-actin gene was used for normalizing the iNOS expression and data represent the Mean ± SE of two independent experiments, and the statistical significance was calculated using Student's *t*-test (N = 7 for each bar represented).

2.9. RNA Isolation and Real Time-PCR

Total RNA was isolated from PBMC of plasmodium (*P.vivax* and *P.falciparum*) infected and healthy subjects using TRI reagents (Ambion, Austin, TX, USA) following manufacturer's guideline and RT-PCR was used to determine the induction of iNOS expression. cDNA was synthesized using an iScript cDNA kit (Bio-Rad) following the manufacturer's protocol. RT-PCR was performed in the IQ5 thermal cycler (Bio-Rad) using the iQ SYBR Green I Supermix (Bio-Rad). The PCR program and primers for iNOS and β-actin were used as previously reported [39,40]. Each reaction contained 10 μl IQ SYBR Green Supermix reagent (Bio-Rad), 10 ng cDNA, and 300 nM each gene-specific primer in a final volume of 20 μl. All RT-PCRs were performed in duplicate. Data were analyzed using the $\Delta\Delta C_T$ method with β-actin used as normalizer.

2.10. Ethics statement and subject consent

All human blood samples used in this study were collected after obtaining written informed consent from all study participants under protocol 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. The participant clinical details and individual demographic information were recorded by our technician in the hospital register as well as in the data register; those who consented to participate in our study and give blood sample for further investigation in addition to malaria blood slide examination. The protocol was approved from IEC, VBU having memo no. VBU/R/885/2012 dated 05-06-2012. The clinical study and experimental procedures were well in accordance with the relevant guidelines and also very much in compliance with the journal's policy.

2.11. Statistical analysis

Data were entered in MS-Excel and analysis 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. The biochemical data are expressed as Mean ± SE. The mean of the parameters for malaria patients and healthy subjects were compared by using Student's *t*-test. Data analysis was performed nonparametrically. The differences were considered significant when $p < 0.05$.

2.12. Calculation of genetic association strength

The model used for risk assessment was the logistic regression adjusted for gender and age. Odd ratios (ORs) and 95% Confidence Intervals (CI) for malarial isolates of each genotype were calculated with logistic regression to quantitatively assess the degree of association and were used to compare categorical variables. Haplotypes frequencies and the extent of association, i.e. the Lewontin's coefficient (*D'*) and squared correlation coefficient (r^2) for pair wise linkage disequilibrium (LD) of the −954G/C and −1173C/T polymorphism were calculated by SNP Alyze software (Version 3.1; Dynacom, Mobara-shi, Japan). The differences observed between the genotype and allele frequency when compared with healthy subjects and the risk factors for either *P. falciparum* or *P. vivax* were evaluated by univariate analysis and then adjusted for significant predictors in multivariate analysis. All tests were conducted at the $P < 0.05$ level of significance.

Table 2
Nitric oxide concentration during malaria infection and effect of axillary temperature on nitric oxide content as compared to healthy subjects.

Parameters	*P. vivax* Mean ± SE (Range)	*P*	Healthy Subjects Mean ± SE (Range)	*P*	*P. falciparum* Mean ± SE (Range)
Sample Size (N)	210		210		110
Nitric Oxide (μM)	33.5 ± 1.1 (10.3–83)	*0.0001*	21.4 ± 1.1 (1.4–68.9)	*0.0001*	34.2 ± 1.1 (12–78.3)

Stratified Axillary (Body) Temperature at the time of Sampling

(°F)	< 99 Mean ± SE (Range)	*P*	> 99–100 Mean ± SE (Range)	*P*	> 100–103 Mean ± SE (Range)	*P*	Healthy Subjects Mean ± SE (Range)
Sample Size	120		117		83		110
Nitric Oxide (μM)	36.4 ± 2.1 (10.9–83)	*0.0001*	34.6 ± 1.9 (10.3–71.5)	*0.0006*	35.8 ± 2.6 (12–74.8)	*0.0007*	22.5 ± 1.8 (5.6–65.4)

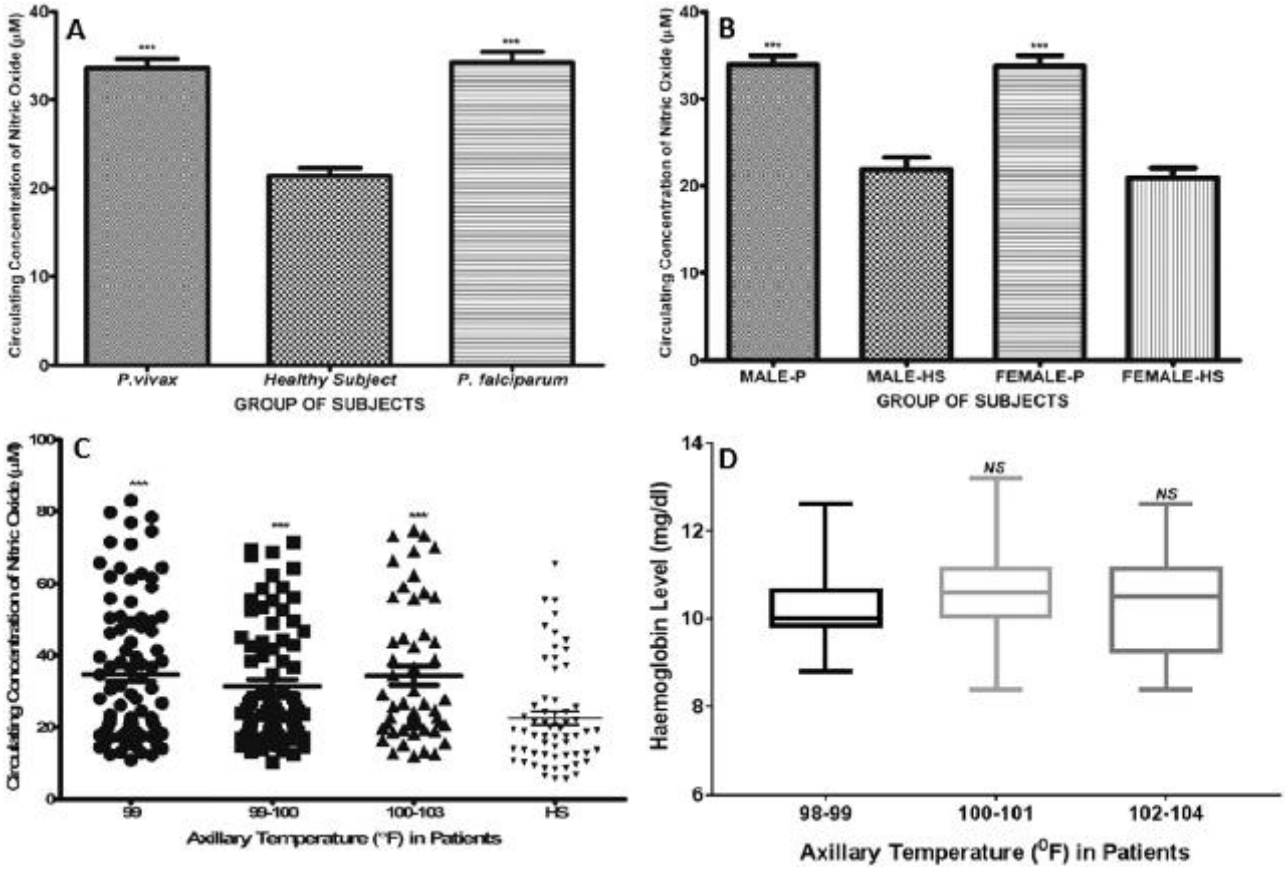

Fig. 1. Circulating concentration of nitric oxide in (A) *P. vivax* and *P. falciparum* infected subjects as compared to healthy subjects (B) *Plasmodium* infected male and female as compared to healthy counterpart (C) Axillary temperature based stratified group of plasmodium infected subjects as compared to healthy group. (D) Level of Haemoglobin in axillary temperature based stratified plasmodium infected subjects. Data is presented as mean and error bar represent the plus or minus SE *p ≤ 0.01; **p ≤ 0.001; ***p ≤ 0.0001 compared with healthy subjects by one-way analysis using Dunn's multiple comparison test and unpaired 't' test with Welch correlation through Graphpad Prism version 5.0.

3. Results

3.1. Nitric oxide content in stratified group of malarial patients and healthy sera samples

A total of 530 subjects (210 *P.vivax* infected, 110 *P. falciparum* infected and 210 healthy subjects) were included in the biochemical study group and considered for analysis. Table 2 shows the serum nitric oxide profiles of subjects with malarial infection. We observed elevated nitric oxide content in overall malaria infected subjects as compared to healthy subjects as shown in Fig. 1A, whereas, marginally higher concentration of nitric oxide was observed in *P. falciparum* infection relative to *P. vivax* infection; the differences in concentration were significant as compared to healthy subjects, as shown in Table 2. We also analyzed the gender specific concentration of nitric oxide in malarial subjects, as shown in Fig. 1B. Although, the level of nitric oxide were higher in both the genders (male and female) as compared to healthy subjects and differences in concentration were significant, surprisingly, we observed almost similar concentration of nitric oxide between the genders in both infected and healthy subjects. Further, in view of a prominent and potent sign & symptom based diagnostic marker and exclusive prevalence of *P. vivax* in the investigated region, we evaluated the impact of stratified axillary (body) temperature on nitric oxide concentration, as shown in Fig. 1C. We observed higher concentration of nitric oxide in all the three stratified groups of patients based on body temperature as compared to healthy subject's body temperature and differences in concentration were significant, as shown in Table 2. Interestingly, we found marginally lower concentration of nitric oxide in 99–100°F axillary temperature group as compared to other groups (99 and 100–103°F) and differences in the content were significant. Further, we also evaluated the haemoglobin concentration based on stratified axillary temperature and observed higher haemoglobin level in higher body temperature range group (101–104°F) of subjects as compared to non-fever and nominal fever (98–99°F) range group of subjects. However, the differences in axillary temperature were non-significant, as shown in Fig. 1D.

3.2. Total antioxidant capacity and reactive oxygen species content in sera samples and PBMC of malarial patients and healthy subjects

Both, total antioxidant capacity (TAC) and reactive oxygen species (ROS) are interdependent components of the cascade regulating the redox metabolism of normal physiology of human system and have potentially dicotomic implications in several diseases including malarial pathology and protection. Considering the indispensable significance and correlation with nitric oxide content and genetic polymorphism in our investigated population, we evaluated the TAC and ROS content in *P. falciparum* and *P. vivax* infected subjects. We observed mean lower total antioxidant content in *P.vivax* (149.1 mM) and *P. falciparum* (133.2 mM) as compared to healthy subjects (437.3 mM). However, higher TAC content was found in *P. vivax* compared to *P. falciparum* infection as shown in Fig. 2A. Similarly, we found mean lower ROS content in *P. falciparum* (166 RFU) and *P.vivax* (220 RFU) infection as compared with healthy subjects (313.7 RFU) as shown in Fig. 2C. However, higher ROS content was observed in vivax infection as compared with falciparum infection.

We also evaluated mutation specific antioxidant capacity and ROS content at both iNOS-954GC/CC and iNOS-1173CT/TT positions in *P.*

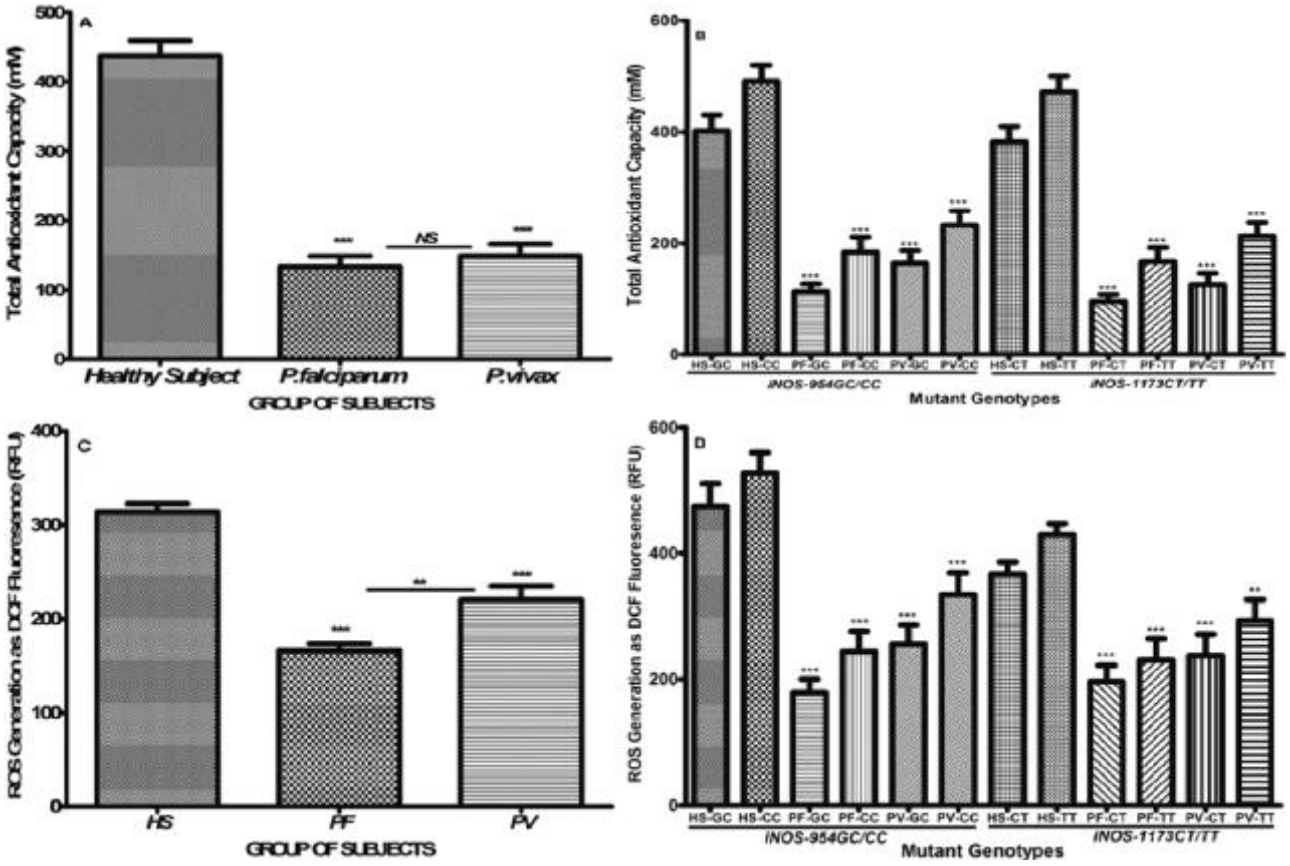

Fig. 2. (A) Circulating concentration of total antioxidant capacity (TAC) content in *P. falciparum* (PF), *P. vivax* (PV) infected and in healthy subjects (HS). (B) Polymorphisms specific level of TAC content in mutant genotypes of malaria infected and in healthy subjects. (C) Circulating concentration of reactive oxygen species (ROS) content in *P. falciparum* (PF), *P. vivax* (PV) infected and in healthy subjects (HS). (D) Polymorphisms specific level of ROS content in mutant genotypes of malaria infected and in healthy subjects. Data is presented as mean and error bar represent the plus or minus SE *p ≤ 0.01; **p ≤ 0.001; ***p ≤ 0.0001 compared with healthy subjects by two-way analysis using Dunn's multiple comparison test and paired 't' test through Graphpad Prism version 5.0.

falciparum and *P. vivax* as compared with mutant genotypes of healthy subjects and found significantly lower antioxidant content in *plasmodium* infection as shown in Fig. 2B as well as significantly lower reactive oxygen species content in *plasmodium* infection as shown in Fig. 2D. However, higher TAC and ROS content was observed in vivax infection compared to falciparum at both iNOS-954G/C and iNOS-1173C/T mutation.

3.3. Polymorphism at the iNOS-954G/C and iNOS-1173C/T and distribution of iNOS −954G/C and −1173C/T genotype in malarial patients

The amplified PCR product for the iNOS-954G→C polymorphism was 573 base pairs. The iNOS-954 polymorphism was screened by the Restriction Fragment Length Polymorphism using 1.5 U of Bsa1 (New England Bio Labs, Cambridge, UK) restriction endonucleases in 10 µl of the PCR product in a total reaction volume of 20 µl and the restricted PCR fragments were separated on a 3% agarose gel. The gel demonstrated two fragments of 437 and 136 base pair for the homowild (GG) genotype, one fragment of 573 base pair for the homomutant (CC) genotype and three fragments of 573, 437, 136 base pairs for the heteromutant (GC) genotype. The amplified PCR products of the iNOS-1173C and iNOS-1173 T alleles were of 131 and 102 base pairs, respectively. The genotype distributions for iNOS promoter polymorphism at position −954G/C and −1173C/T in *vivax* and *falciparum*-infected patients were compared with healthy subjects and detailed results are shown in Table 3. At iNOS-954 position, the C allele was observed in 23.1% and 16.5% of vivax and falciparum subjects, respectively as compared to 8.6% in healthy subjects. Similarly, at iNOS-1173 position the prevalence of T allele was 23.5% and 25.3% in vivax and falciparum subjects, respectively as compared to 12.3% in healthy subjects.

3.4. Association between iNOS genotypes and risk of malaria

Using the iNOS −954 GG and iNOS −1173CC as the reference group, we observed that a statistically significant increased risk of malaria was associated with the −954 and −1173combined (GC + CC) and (CT + TT) genotypes in both *P. vivax* and *P. falciparum*, as shown in Table 3. However, highest risk was observed to be associated with *iNOS* −954 homozygous variant CC (adjusted OR = 2.54; 95% CI = 0.72–2.63), followed by combined (GC + CC) variant (adjusted OR = 1.92; CI = 0.71–1.98) and heterozygous variant GC (adjusted OR = 1.73; CI = 0.25–1.82) in *P. vivax* followed by *P. falciparum* CC (adjusted OR = 1.74; 95% CI = 0.93–1.76), followed by combined (GC + CC) variant (adjusted OR = 1.68; CI = 0.84–2.25) and heterozygous variant GC (adjusted OR = 1.46; CI = 0.23–1.26). Similarly, highest risk was observed to be associated with *iNOS* −1173 homozygous variant TT (adjusted OR = 1.94; 95% CI = 0.22–1.98), followed by combined (CT + TT) variant (adjusted OR = 1.72; CI = 0.71–1.98) and heterozygous variant CT (adjusted OR = 1.63; CI = 0.25–1.82) in *P. vivax* followed by *P. falciparum;* TT (adjusted OR = 1.63; 95% CI = 0.93–1.76), combined (CT + TT) variant (adjusted OR = 1.75; CI = 0.84–2.25) and heterozygous variant CT (adjusted OR = 1.57; CI = 1.23–2.96), shown in Table 3.

Table 3
Genotypic frequencies of the *iNOS* polymorphism in cases and controls and their associations with risk of *Plasmodium vivax* and *Plasmodium falciparum*.

Genotypes	Cases		Controls		Adjusted OR (95% CI)	*P*
iNOS – 954 (*P. vivax*)	N = 119	%	N = 110	%		
GG	78	65.54	95	86.36	1.00	0.006
GC	27	22.68	11	10	1.73 (0.25–1.82)	
CC	14	11.76	4	3.63	2.54 (0.72–2.63)	
GC + CC	41	34.44	15	13.63	1.92 (0.71–1.98)	0.027
C allele		23.11		8.63		0.043
iNOS- 954 (*P. falciparum*)	N = 91	%	N = 110	%		
GG	68	74.72	95	86.36	1.00	0.007
GC	16	17.58	11	10	1.46 (0.23–1.26)	
CC	7	7.69	4	3.63	1.74 (0.93–1.76)	
GC + CC	23	25.27	15	13.63	1.68 (0.84–2.25)	0.034
C allele		16.48		8.63		0.026
iNOS – 1173 (*P. vivax*)	N = 119	%	N = 110	%		
CC	72	60.5	81	82.72	1.00	0.036
CT	34	28.57	21	10	1.63 (0.25–1.82)	
TT	13	9.24	8	7.27	1.94 (0.22–1.98)	
CT + TT	47	37.81	29	17.27	1.72 (0.71–1.98)	0.027
T allele		23.52		12.27		0.043
iNOS- 1173 (*P. falciparum*)	N = 91	%	N = 110	%		
CC	54	59.34	81	82.72	1.00	0.004
CT	28	30.76	21	10	1.57 (1.23–2.96)	
TT	9	9.89	8	7.27	1.63 (0.93–1.76)	
CT + TT	37	17.1	29	17.27	1.75 (0.84–2.25)	0.023
T allele		25.27		12.27		0.026

3.5. Association between serum nitric oxide concentration and iNOS polymorphism in malaria

Furthermore, to explore the relationship between the iNOS-954 and iNOS-1173 transition polymorphism and the serum NO activity in infection specific patients i.e. in *vivax* and *falciparum*, we evaluated serum NO activity in different genotype of iNOS-954 and iNOS-1173 as shown in Table 4. We observed that iNOS-954 genotypes (GG, GC and CC) group has significantly higher serum NO activity in case of *vivax* (Fig. 3A), *falciparum* (Fig. 3B) and overall *plasmodium* (Fig. 3C) infection. In *P.vivax,* we observed increased nitric oxide content in all the genotypes and the differences in the nitric oxide content were found to be statistically significant ($P = 0.0001$, $P = 0.01$ and $P = 0.04$ for GG, GC and CC, respectively). Most interestingly, the risk associated genotype i.e. CC show significantly highest nitric oxide concentration (63.77 μmole) as compared to all the genotypes in patients. Similarly, in case of *falciparum* infection, we found significantly elevated nitric oxide in GG and CC ($P = 0.0001$ and $P = 0.03$, respectively) genotype as compared to healthy subjects whereas, the NO content was almost similar in case of GC genotype, shown in Fig. 3B. As expected, the NO content in the risk associated genotype (CC) in *falciparum* infection was found to be highest (65.29 μmole) as compared to all the genotypes in *vivax* as well as *falciparum* subjects, shown in Fig. 3A & B and Table 4. In addition, we analyzed the effect of iNOS-954 transition polymorphism on NO content during overall plasmodium infection and interestingly observed that all the genotypes had significantly higher ($P = 0.0001$, $P = 0.01$, and $P = 0.03$ for GG, GC, and CC genotypes, respectively) nitric oxide level as compared with healthy subjects as shown in Fig. 3C. When effects of this polymorphism on NO content were compared between the *vivax* and *falciparum* infection, we observed significant association between all the genotypes, however, NO content was higher in GG and GC genotype in *vivax* as compared to *falciparum* whereas NO content was higher in *falciparum* CC genotype as compared with *vivax* infection as shown in Fig. 3D.

In regard to iNOS-1173 transition polymorphism, our observations showed that compared with healthy subjects, the iNOS-1173 genotypes

Table 4
Association of iNOS −954G/C and iNOS −1173C/T polymorphism with nitric oxide level in malarial patients as compared to healthy subjects.

Genotype(−954 G/C)	**GG**	***P*[a]**	**GC**	***P*[a]**	**CC**	***P*[a]**
P. vivax	N = 76		N = 24		N = 25	
Nitric Oxide (μmole)	22.15 ± 0.81	*0.0001*	47.13 ± 1.70	*0.01*	63.77 ± 2.02	*0.04*
Healthy Subjects	N = 85		N = 18		N = 7	
Nitric Oxide (μmole)	11.26 ± 0.56		41.28 ± 1.67		57.14 ± 2.83	
P. falciparum	N = 55		N = 22		N = 14	
Nitric Oxide (μmole)	19.44 ± 0.63	*0.0001*	40.98 ± 1.29	*NS*	65.29 ± 2.07	*0.03*
Plasmodium Infection	N = 125		N = 46		N = 39	
Nitric Oxide (μmole)	20.96 ± 0.81	*0.0001*	44.19 ± 1.16	*0.01*	64.32 ± 1.48	*0.03*
Genotype (−1173C/T)	**CC**	***P*[a]**	**CT**	***P*[a]**	**TT**	***P*[a]**
P. vivax	N = 82		N = 26		N = 11	
Nitric Oxide (μmole)	24.73 ± 0.63	*0.0001*	40.88 ± 0.89	*0.009*	56.32 ± 1.44	*NS*
Healthy Subjects	N = 91		N = 11		N = 8	
Nitric Oxide (μmole)	20.05 ± 0.4		32.99 ± 0.82		52.44 ± 1.25	
P. falciparum	N = 58		N = 25		N = 8	
Nitric Oxide (μmole)	27.28 ± 0.48	*0.0001*	48.13 ± 1.01	*0.0001*	65.75 ± 1.08	*0.0001*
Plasmodium Infection	N = 140		N = 51		N = 19	
Nitric Oxide (μmole)	26.01 ± 0.44	*0.0001*	44.43 ± 0.83	*0.0001*	60.29 ± 1.43	*0.003*

[a] When compared with healthy subjects.

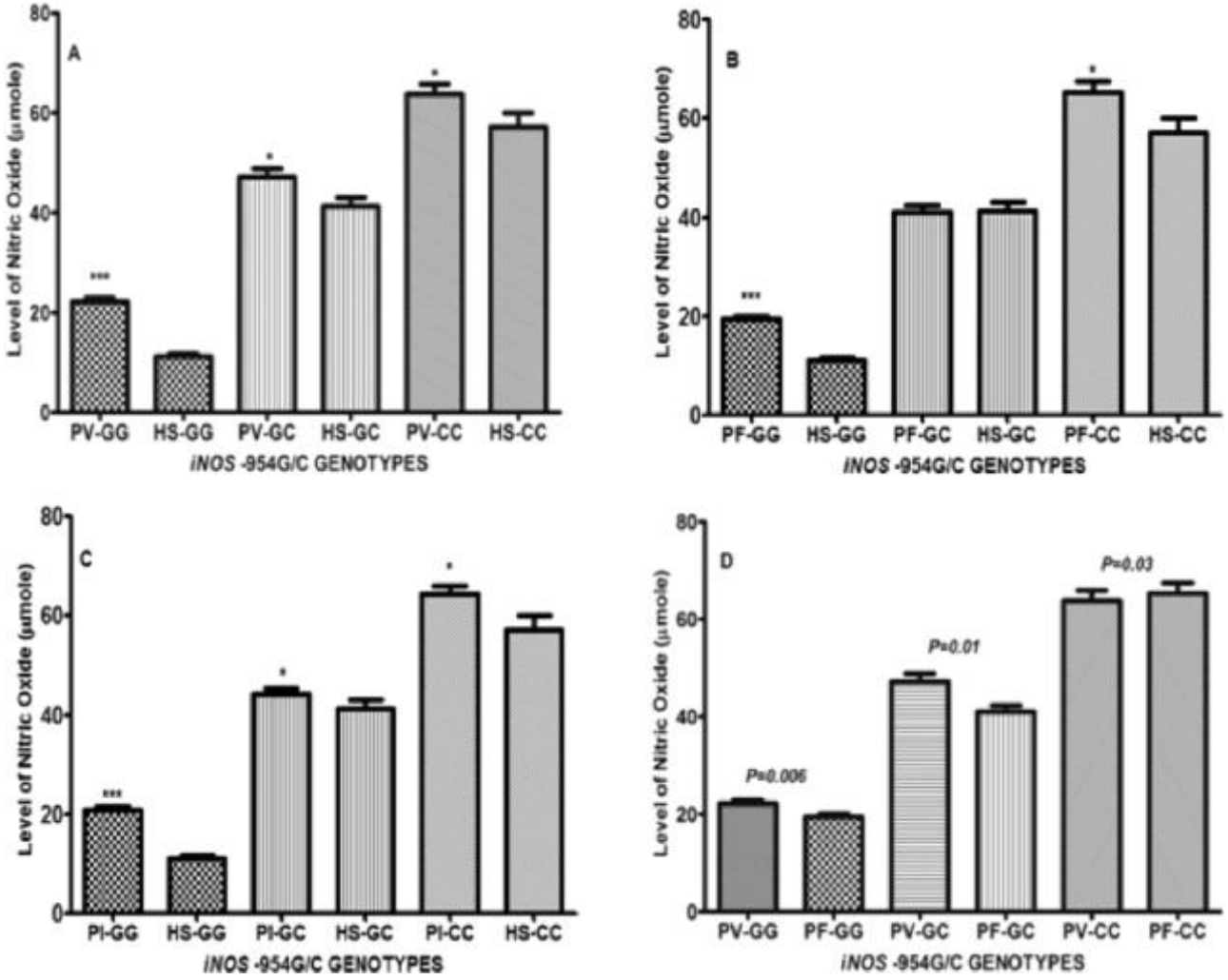

Fig. 3. (A) Effect on nitric oxide level in *P. vivax* (PV) as compared with healthy subjects (HS) based on iNOS-954G/C polymorphism. (B) Effect on nitric oxide level in *P. falciparum* (PF) as compared with healthy subjects (HS) based on iNOS-954G/C polymorphism.(C) Effect on nitric oxide level in plasmodium infection (PI) as compared with healthy subjects (HS) based on iNOS-954G/C polymorphism. (D) Comparative effect on nitric oxide level in *P. vivax* (PV) as compared with *P. falciparum* (PF) based on iNOS-954G/C polymorphism. Data is presented as mean and error bar represent the plus or minus SE *p ≤ 0.01; **p ≤ 0.001 and ***p ≤ 0.0001 compared with healthy subjects by one-way analysis using Dunn's multiple comparison test and unpaired 't' test with Welch correlation through Graphpad Prism version 5.0.

(CC, and CT group) had significantly higher serum NO activity (as shown in Fig. 4A) in case of vivax infection ($P = 0.0001$, and $P = 0.009$ for CC, and CT, respectively) and interestingly the risk associated genotype i.e. TT showed highest nitric oxide activity (56.32 µmole) as compared to CC and CT genotypes in patients and even more than the risk associated genotype of the healthy subjects (52.44 µmole) but the difference was found to be non-significant. However, in case of *falciparum* infection, we found significantly elevated nitric oxide in all the genotypes ($P = 0.0001$, $P = 0.0001$, and $P = 0.0001$ for CC, CT, and TT respectively) as compared with healthy subjects as shown in Fig. 4B. In addition to this, we evaluated the effect of iNOS-1173 transition polymorphism on NO content during overall plasmodium infection and interestingly observed that all the genotypes had significantly higher ($P = 0.0001$, $P = 0.0001$, and $P = 0.003$ for CC, CT, and TT genotypes, respectively) nitric oxide level compared with healthy subjects as shown in Fig. 4C. When the effect of polymorphisms on NO content was compared between the vivax and *falciparum* infection, we observed significant association between all the genotypes, although NO content was higher in *falciparum* infection as compared to *vivax* infection in all the genotypes as shown in Fig. 4D.

3.6. Association between iNOS haplotypes and risk of malaria

The Linkage Disequilibrium tests (LD) showed that the two polymorphisms of *iNOS* at position-954G/C and −1173C/T were in highly significant LD ($D' = 0.6648$, $r^2 = 0.4003$, $p < 0.0001$) in the investigated subjects. Since, −954G/C and −1173C/T polymorphisms were found to be in highly significant LD; hence case-control haplotypes analysis was performed. Maximum likelihood procedure suggested that all the possible haplotypes, such as 954G:1173C, 954C:1173T, 954C:1031C and 954G:1173T, respectively, in both the polymorphisms significantly differed between patients and healthy subjects ($p = 0.01$, $p = 0.001$, $p = 0.002$, $p = 0.001$, respectively). However, the dominance of 954G:1173C alleles associated haplotypes was observed in patients (0.7426) compared to healthy subjects (0.3422). It is interesting to report that the distribution of novel 954C:1173T alleles associated haplotypes were significantly higher in case of healthy subjects (0.4561) as compared to patient group (0.3371). The other two haplotypes (954C:1173C and 954G:1173T, respectively) were also significantly different but under-represented in the patients (0.061, 0.036, respectively) as compared to healthy subjects (0.171, 0.138, respectively).

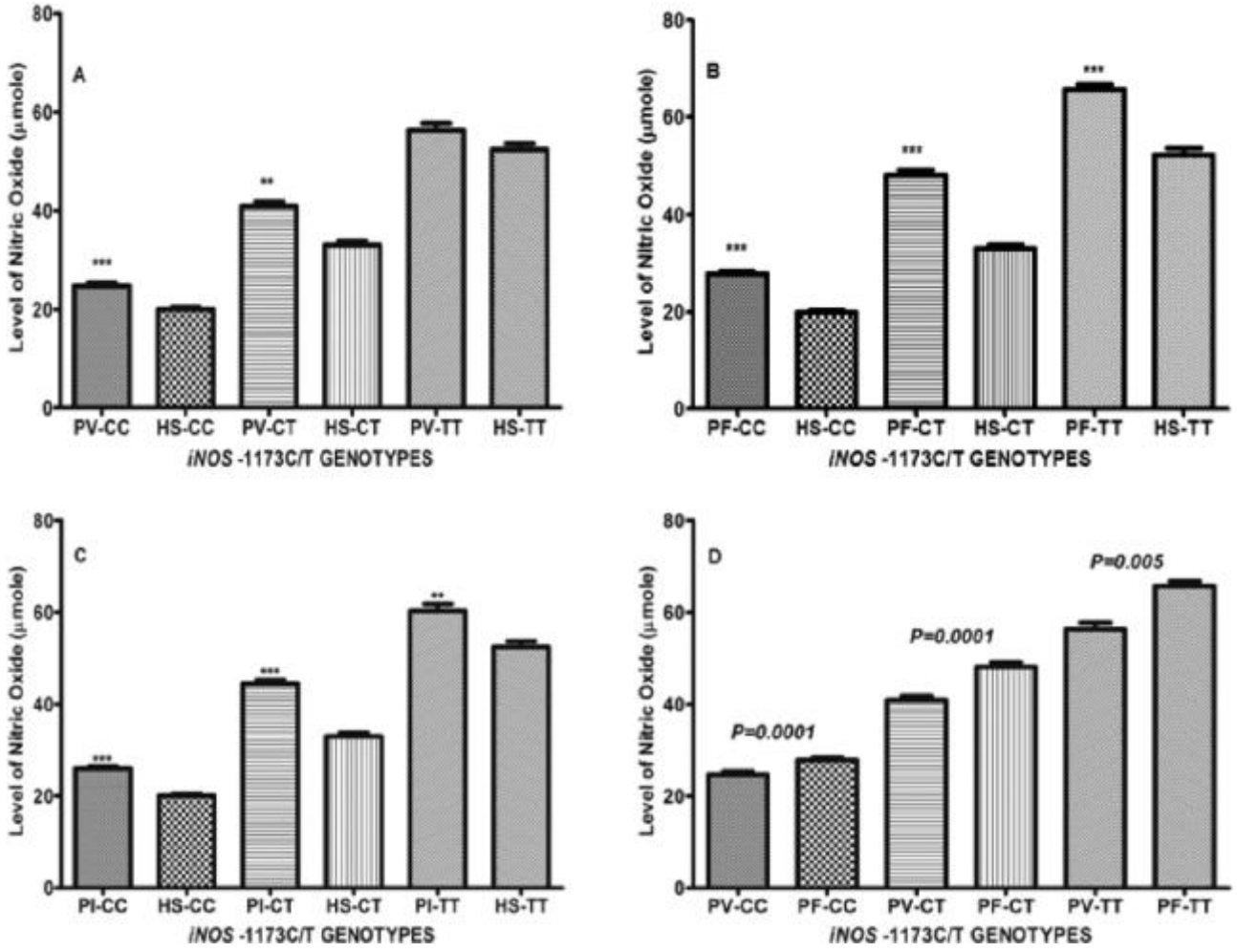

Fig. 4. (A) Effect on nitric oxide level in *P. vivax* (PV) as compared with healthy subjects (HS) based on iNOS-1173C/T polymorphism. (B) Effect on nitric oxide level in *P. falciparum* (PF) as compared with healthy subjects (HS) based on iNOS-1173C/T polymorphism.(C) Effect on nitric oxide level in plasmodium infection (PI) as compared with healthy subjects (HS) based on iNOS-1173C/T polymorphism. (D) Comparative effect on nitric oxide level in *P. vivax* (PV) as compared with *P. falciparum* (PF) based on iNOS-1173C/T polymorphism. Data is presented as mean and error bar represent the plus or minus SE *p ≤ 0.01; **p ≤ 0.001 and ***p ≤ 0.0001 compared with healthy subjects by one-way analysis using Dunn's multiple comparison test and unpaired 't' test with Welch correlation through Graphpad Prism version 5.0.

3.7. iNOS expression in the P. vivax and P. falciparum infected subjects

The inducible NOS are actively engaged in defence against various parasites and parasite induced host factors by inducing nitric oxide, inflicting oxidative damage, regulating gene expression and has been characterized as potential metabolic component in determining the course of diseases in a range of infections. In view of the critical role of the iNOS/NO pathway in pathophysiology and host defence in infectious disease patients including tropical diseases, we investigated whether plasmodium infection has any influencing effect on the expression of iNOS/NO pathway compared to healthy subjects. Additionally, we also investigated the impact of genetic polymorphism on the functional expression of iNOS. In order to elucidate the infection and mutation induced activation mechanisms and biological specificity of iNOS/NO pathway; we analyzed the expression of iNOS protein and RNA from PBMCs of plasmodium infected, healthy and polymorphism stratified subjects by employing western blot and qRT-PCR. We resolved and blotted the total protein as per the Fig. 5A from plasmodium infected and healthy subjects and observed higher expression of iNOS protein in *P. Falciparum* and *P.vivax* infected subjects as compared to healthy subjects, as shown in Fig. 5B. Interestingly, we observed differential expression of protein in *P. falciparum* and *P.vivax* infected subjects and differences were statistically significant ($P = 0.0004$), as shown in Fig. 5C. Furthermore, western blot analysis revealed almost 2.4 and 1.8 fold increase of iNOS protein in *P. vivax* and *P. falciparum* infected subjects, respectively, as compared to healthy subjects and differences were significant, as shown in Fig. 5C.

Based on the results so far and precisely, observations of genetic polymorphisms and differential protein expression, we conceptualize that plasmodium specific infection modulates the iNOS/NO pathway via perturbed antioxidant and reactive oxygen species components and they finely regulate the iNOS/NO expression during malaria pathology. To further consolidate and substantiate our observation regarding implications of mutation specific transcriptional induction of iNOS/NO expression; we analyzed the mRNA expression in both infection and mutation specific stratified samples as compared with healthy subjects. We observed plasmodium infection specific increased expression of iNOS mRNA as compared with healthy subjects, as shown in Fig. 6A. RT-PCR revealed 2.5 and 1.5 fold increase expression in *P. vivax* and *P. falciparum* infected subjects, respectively, as compared to healthy subjects and differences were significant, as shown in Fig. 6A.

Further, we also evaluated mutation specific iNOS mRNA expression in both iNOS-954GC/CC and iNOS-1173CT/TT polymorphism in *P. falciparum* and *P.vivax* as compared with mutant genotypes of healthy subjects. We observed increased and differential expression in both mutations and infections; and differences were found significant, as shown in Fig. 6B and C, respectively. Though, relatively higher expression was observed in *P.vivax* infection as compared to *P. falciparum*

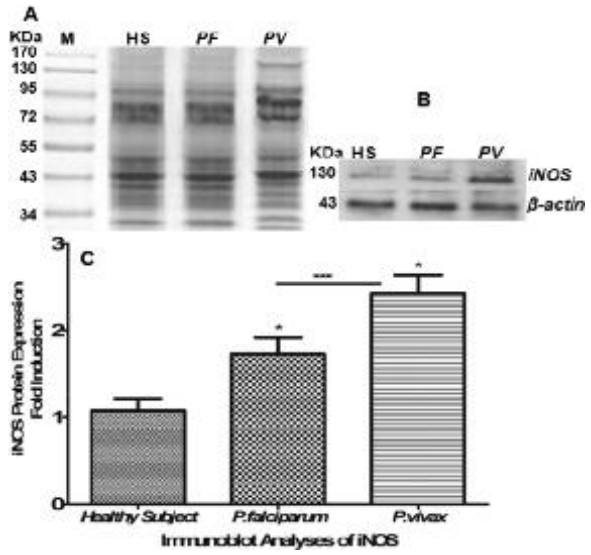

Fig. 5. (A) SDS-PAGE profile of total protein for iNOS resolution from *Plasmodium*-infected isolates compared with healthy subjects. Lane 1, protein marker (M); Lane 2, homogenate from healthy subjects (HS); lane 3, homogenate from *P. falciparum* (PF); lane 4, homogenate from *P. vivax* (PV); (B) Immunoblot of expressed iNOS protein in *P. falciparum* and *P. vivax* as compared with healthy subjects; (C) Analyses of immunoblot as the mean fold increase relative to the quantity of iNOS induced and normalized to the level of cellular actin, as assessed by densitometry. The data represent the mean ± SE of two independent experiments (N = 7 for each column) and the statistical significance *P < 0.05; **P < 0.0005 compared with healthy subjects was calculated using two-way analysis using Tukey's multiple comparison test through GraphPad Prism version 5.0.

infection in both the mutations. Additionally, as observed, anticipated and point of interest as well that those mutant genotypes (hetero and homo mutants) in both infection and positions have shown differential higher expression of iNOS and overall the differences were significant, as shown in Fig. 6D.

The above data validate the results of qualitative estimation and immunoblot, that polymorphism promotes the expression of iNOS in response to *Plasmodium* infection. These results collectively suggest that the presence of both hetero and homo mutant genotype enhances iNOS expression in response to *Plasmodium* infection in general and particularly more in case of *P. vivax* infection, resulting in increased production of nitric oxide and critically regulating other orchestrating components of the cascades like reactive oxygen and nitrogen intermediates associated with malarial pathology.

3.8. Association among nitric oxide, axillary temperature (body temperature) and age in malaria infected subjects

In view of the pathological and physiological relevance of body temperature during *vivax* infection, we investigated the prospective clinical correlation among nitric oxide, axillary temperature and age in infected subjects with an intention to explore and establish the possibility of biological association. We observed negative and significant association (p = 0.0001 and p = 0.0001) of body temperature with nitric oxide but weakly correlated ($r^2 = 0.003$ and $r^2 = 0.04$ respectively) both in *P. vivax* and *P. falciparum* malarial as compared to healthy subjects, as shown in Fig. 7B, F, and D, respectively. Interestingly, a tendency towards a marginal decrease in concentration of NO level with increase in axillary temperature was observed both in *plasmodium* infection and healthy subjects; though in *falciparum* decreasing concentration of NO level is higher than *vivax* infection as shown in Fig. 7.

Further, we observed marginally negative and significant association ($p = 0.0001$) of age with nitric oxide but weakly correlated ($r^2 = 0.04$) in *vivax* infected subjects, although no clear cut trend was observed, as shown in Fig. 7A. In case of falciparum infected subjects, almost similar observation was found and correlation was also non significant, as shown in Fig. 7E. Conversely, in the case of healthy subjects, we observed marginally positive and significant (p = 0.0001) association of age with nitric oxide but weakly correlated ($r^2 = 0.004$), exhibiting a trend of marginal increase in the concentration of nitric oxide with increase in age, as shown in Fig. 7C.

4. Discussion

Malaria patients show variable degree of clinical manifestations which appears to be mediated by perturbation in the orchestration of complex biochemical pathways and immunological cascades of the host in response to parasites or their toxins. Among the various pathological mediators, nitric oxide is an important mediator and critical signalling molecule for malaria which is constitutively expressed by the host machinery during the complex life cycle of *Plasmodium* [41]. However, nitric oxide and related intermediates have dicotomic role in malaria so far; some reported their involvement in development of disease severity [13–17], whereas, others have shown protective role [18–23]. Due to these contradictory observations, this wonder molecule remains enigmatic for elucidation and intriguing parasitologist to investigate whether NO is protecting or promoting malarial pathology [42]. In view of understanding the disease progression and factors influencing the clinical outcome of an infection which is crucial for early diagnosis and prevention strategy; we evaluated the circulating concentration of nitric oxide, association of transition polymorphism, functional and mechanistic impact of transition polymorphism on differential expression of nitric oxide synthase in *P. vivax* and *P. falciparum* infected subjects. We observed significantly higher nitric oxide in both *vivax* and *falciparum* infected subjects as compared to healthy subjects, although, marginally higher NO concentration was observed in *vivax* infected subjects as compared to *falciparum* infection. Our findings are in accordance with the observations made by Craig S. Boutlis et al. [35], Lima-Junior JC et al. [43] and Viktoria Jeney et al. [44]. The changes in NO levels and *iNOS* induction in malaria and its involvement in the clearance of malarial parasites apparently varies according to the stage of infection, the degree of parasitemia, the strain of *Plasmodia* and the target organ or tissue being examined [42]. Additionally, observations are also influenced by the coordinated components especially biological mediators, triggering factors, host innate characteristics, parasites virulence and localization and the time of sampling [45,46]. Early increase in NO could stimulate Th1 cell types to produce more mediators (RNI, ROI, O_2) to control parasitemia during infection, whereas late production in liver and spleen appear to have pathological consequences in association with hepatosplenomegaly [47,48]. Malarial infection has the ability to activate the immune cells which causes the release of reactive oxygen species (ROS) with the potency of inducing oxidative damage and cell destruction [49]. In malaria, ROS are generated as byproduct of parasite haemoglobin metabolism in RBCs, NADPH oxidase in phagocytes, and through nitric oxide synthase when the substrate arginine or cofactor tetrahydrobiopterin is lacking [50]; and have dicotomic role ranging from host defense and influencing metabolic cascades to disease severity [51]. We observed lower reactive oxygen species and higher nitric oxide content during plasmodium infection as compared to healthy subjects and this is in accordance with the observations made by Hemmer et al. [52], Sobolewski et al. [53] and, recently Lind et al. [54]. Our observation of lower ROS content in malaria is unconventional and the probable reason which can be attributed to this finding is that the pathway regulating the cyclooxygenase (COX) enzyme complex gets induced through various stimuli including family of lipid mediators at the site of infection and inflammation resulting into the expression of both constitutive COX1 and inducible COX2 forms of COX [54]. In a paracrine fashion, COX1 and COX2 interacts with the upregulated iNOS/NO pathway in disease condition and critically regulate the production of COX2 [55] i.e. expression of enzyme COX2 is reduced in presence of elevated nitric oxide

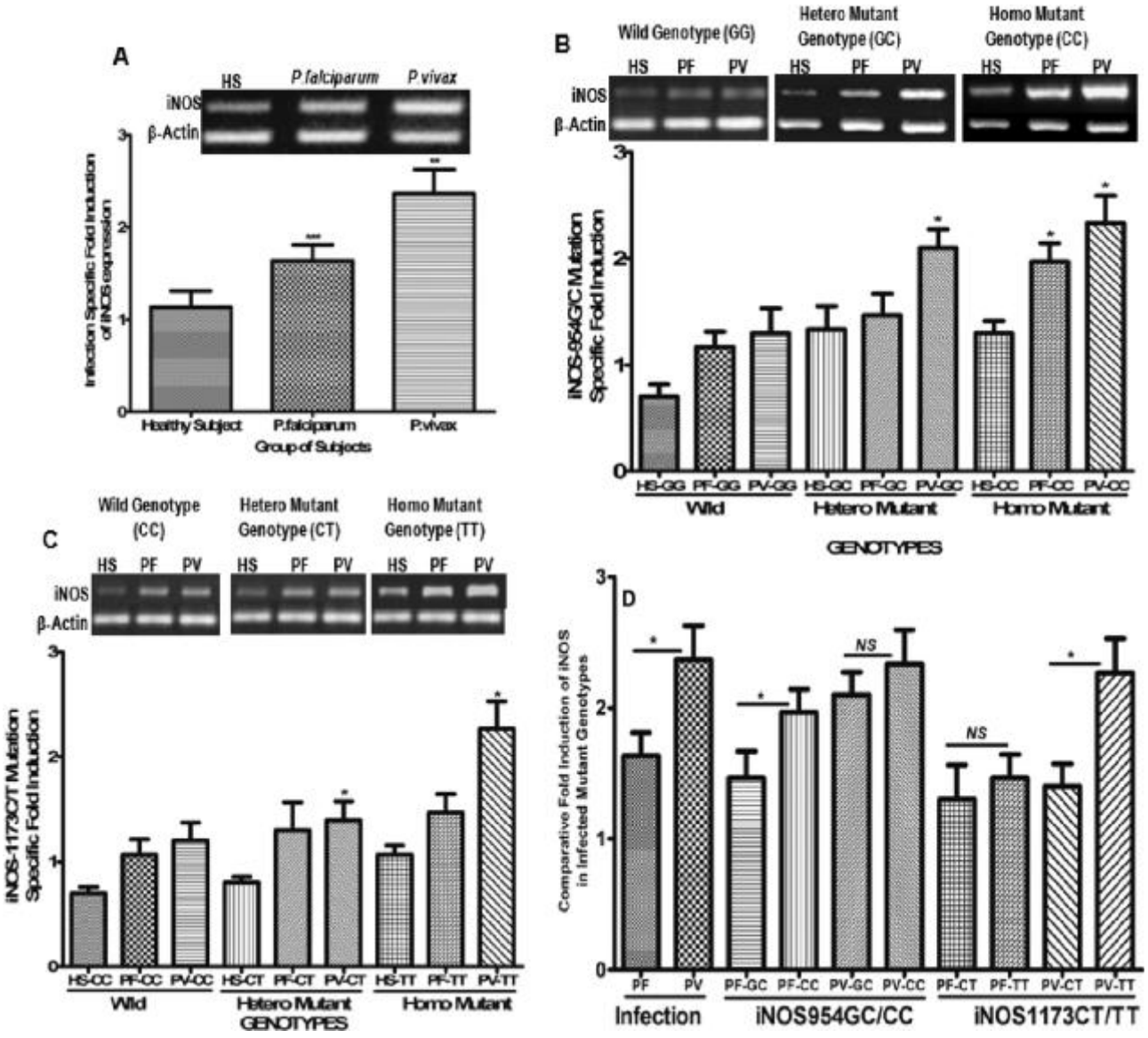

Fig. 6. Fresh PBMCs were processed from healthy subjects (HS) and *P. vivax* (PV) and *P. falciparum* (PF) infected patients for RNA isolation and cDNA. (A) Differential expression of iNOS during *Plasmodium* infection. (B) iNOS-954G/C mutation-specific expression of iNOS; (C) iNOS-1173C/T mutation-specific expression of iNOS; and (D) Relative expression of iNOS in plasmodium species and in mutant genotypes of both transition polymorphisms. Fold induction was calculated using the $\Delta\Delta C_T$ method of qRT-PCR, in which uninfected samples were compared to infected samples relative to β-actin levels as per the cropped gel image placed at the top of the each bar representing. Data are presented as means and error bars represent ± SE from seven isolates. Results are representative of at least three independent experiments. *P < 0.05; **P < 0.0005 compared with healthy subjects by one-way analysis using Tukey's multiple comparison test through GraphPad Prism version 5.0.

level by concentration dependent feed-back inhibition mechanism via the c-Ab1/Arg pathway, resulting into decreased ROS production. Moreover, when NOS activity and NO content are high, O_2^- production is suppressed [56,57] as L-Arg regulate NO production by enhancing *ARG1* transcription and the resultant up regulation and/or over-expression of *ARG1* during infection induces negative feed-back loop mechanism to regulate and suppress its own production via L-Arg pre-treatment, culminating into reduced ROS production [58] and is the likely causal mechanism operating in our observation. Furthermore, to avoid and overcome the oxidative stress and damages and to equilibrate the redox metabolism; plasmodium utilizes the orchestrated components of its antioxidant machinery against the efficient enzymatic host's antioxidant defense system. In order to understand the alterations in redox interplay and predisposition of disease severity in clinical perspective of the host responses to malaria; we evaluated and observed decreased total antioxidant content and increased nitric oxide content in plasmodium infected patients as compared to healthy subjects. Our observation of lower antioxidant is in agreement with the finding of Blair et al. [59], Aghedo et al. [60] and Das et al. [61] who showed its correlation with disease severity. The reason of this observation can be attributed to the excess utilization of host's antioxidants to combat the malaria infection-associated oxidative damages [62]. Degradation of antioxidant enzymes as well as haemoglobin by malaria parasite to produce its own protein has been reported [63] and this might be contributing to the decline in total antioxidant status. The total antioxidant level varied inversely with the severity of malaria indicating that our investigated subjects may have clinical symptoms alike mild/moderate malaria [64,65]. Therefore, our study shows that free radical challenge could induce reduction in antioxidant levels and strengthens the involvement of antioxidants in the defense against ROS induced damages by the parasite. Further, antioxidants have shown significant influence on host's immunity and in neutralizing the adverse effect of free-radicals and associated toxic byproduct. Antioxidants are degraded by malarial parasites to derive amino acids and other essential

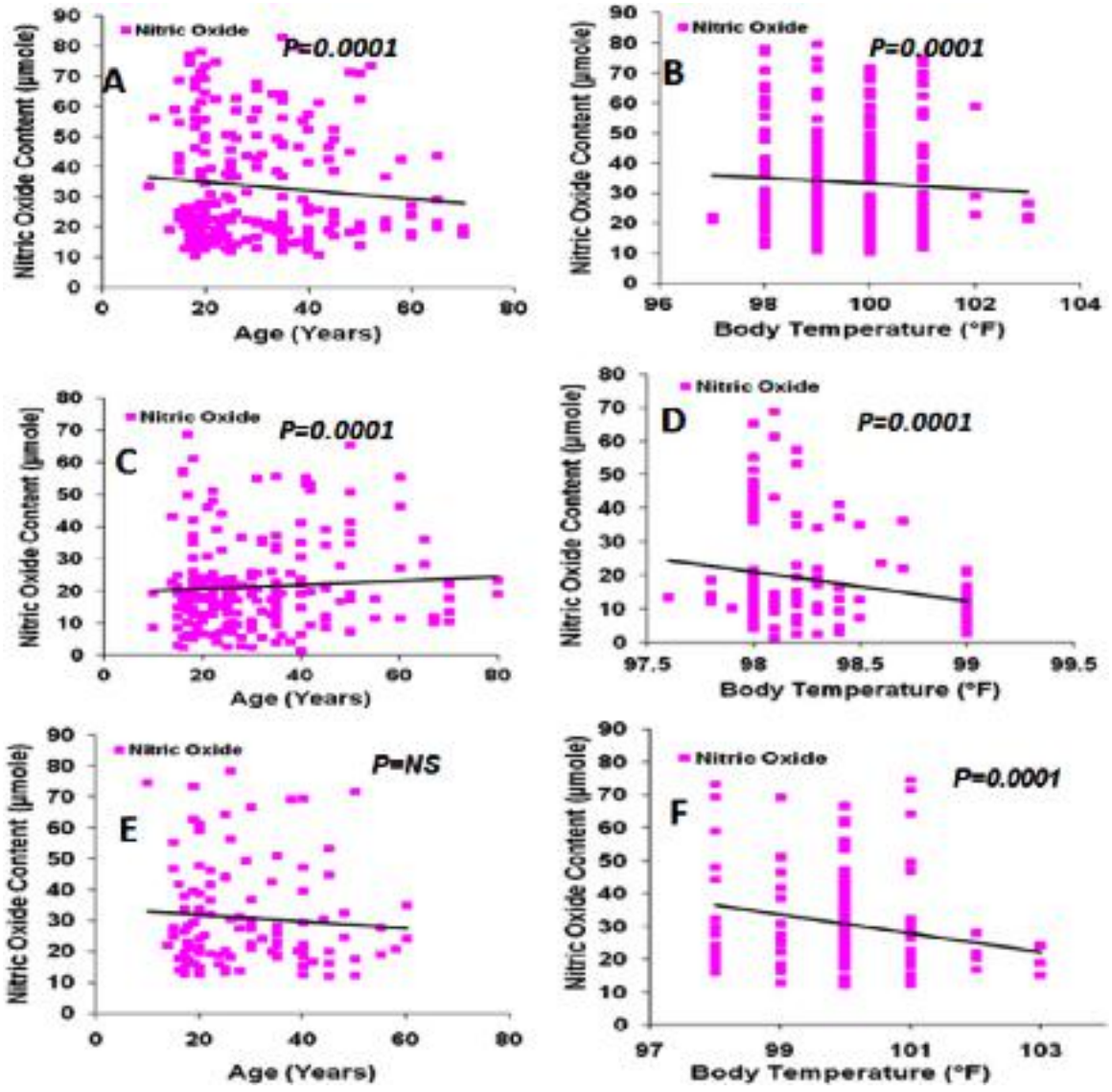

Fig. 7. Association of nitric oxide, axillary temperature (body temperature) and age during *plasmodium* infection. (A) Correlation between nitric oxide concentration and age during *vivax* infection. (B) Correlation between nitric oxide concentration and body temperature during *vivax* infection. (C) Correlation between nitric oxide concentration and age in healthy subjects. (D) Correlation between nitric oxide concentration and body temperature in healthy subjects. (E) Correlation between nitric oxide concentration and age during *falciparum* infection. (F) Correlation between nitric oxide concentration and body temperature during *falciparum* infection. Statistical significance was determined by Student's *t*-test.

molecules for survival as the same may not be replenished by red blood cells due to augmented protein synthesis in diseased condition, and might be one of the plausible reasons behind overall depleted total antioxidant content in the host [66,67].

In genetic polymorphism study, we observed that iNOS-954 GG + GC and iNOS-954 CC genotypes were associated with an increased risk of malaria. The association was more pronounced in patients with *vivax* malaria and in overall *Plasmodium* infection compared to *falciparum* malaria. However, the association was observed to be significant in all the three cases as compared to healthy group. Further, iNOS-1173CC + CT genotypes and the iNOS-1173 TT genotypes were also observed to be associated with an increased risk of malaria, but contrary to the observation of iNOS-954; the association was more pronounced in patients with *falciparum* malaria and in overall *Plasmodium* infection compared to *vivax* malaria. This study does not support a protective association in both type of transition polymorphism; rather it supports significant association with malarial pathology which is in accordance with the previous observations made by other investigators in malaria [31,32]. Our findings further endorses and elucidates the observation of Hobbs et al. regarding association of transition polymorphism at −954 and −1173 position of NOS promoter with malaria disease progression and severity in African population; although genotype distributions and degree of association in both the transition polymorphism vary with the ethnicity [31]. These findings in the investigated population may be potentially limited by the sample size in addition to various other factors like increased recombination near SNP sites, gene conversion, selection involving the 5' and 3' SNP clusters, or epistatic selection. Additionally, geographic variation, susceptibility to malaria and disease phenotype may be attributed to differences in host genetics, parasite strains and malaria epidemiology. The functional significance of these conflicting observations is ambiguous, suggesting that the relationship between NOS2 polymorphism and malaria severity is much more complex than previously described [68–70]. Disparities in allele frequency make an ideal source of spurious association; however they should not be always overlooked as differences in functional alleles of this sort might explain varied response between populations in susceptibility/protection from diseases. The divergent evolutionary histories per locus underlying such allele difference may often be shaped by related infectious diseases that shares related biological mechanisms; hence explaining major

differences between populations for various disease profiles [71]. Therefore, it is clear that comparisons of these parameters between different regions of the genome are highly dependent on factors that may have little to do with the intrinsic mutation rate or the influences of selective pressure on a given region of DNA [70].

Moreover, our findings are pertinent and their interpretation can be attributed to several reasons because the iNOS-954 polymorphism is located in the iNOS promoter region, which could be an activator of gene expression and nitric oxide production [20]. iNOS-954C allele, but not the iNOS-954G allele, may have a higher affinity to a DNA binding protein and thus, translational activity and NOS mRNA levels were increased in the iNOS-954C allele resulting in overall enhanced NOS expression. This rationale is quite well in accordance with our findings of increased iNOS protein and mRNA level both in infection induced as well as mutation specific expression as previously observed by Kun et al. [20], Ascenzi P et al. [72] and Chiwakata CB et al. [73]. This also corroborates with our observation that iNOS-954C allele showed increased NO levels. Although, dicotomic and opposing findings were also reported by some earlier investigators regarding protective role and parasite killing depending upon different cell types from different regions [74], several others opined that transition polymorphisms in promoter region of iNOS gene alters NO level and a higher NOS activity in human PBMCs are associated with uncomplicated malaria [31], severe malaria and clinical complications in addition to activating protective pathways [11,75,76]. NO production and expression of iNOS in *Plasmodium* infection is activated by variety of critical triggering components in the balancing of arginine-nitric oxide pathway, including increased arginase activity and cell-free haemoglobin in plasma, low levels of the NOS substrate arginine and the NOS cofactor tetrahydrobiopterin [77] in addition to multiple mechanisms by which NO bioavailability is critically regulated for impairment of NOS2 expression. Interestingly, it also facilitates alterations of unique monocyte activation phenotypes through PBMC/monocytes that plausibly regulates the effective concentration of nitric oxide, adherence of parasitized RBC to endothelium; a potential regulator of pathophysiological mechanism in malaria disease progression and protection [78,79]. In view of our observation of elevated nitric oxide concentration, expression of NOS in uncomplicated malaria subjects and polymorphic variability in NOS promoter region and to substantiate our findings of significantly associated observation in disease progression, we would like to discuss (though contrary to our findings with a differentiating limitations of study design and population) a very prominent and biologically rational observations made by Anstey NM et al. [11] that nitric oxide production and NOS2 expression are inversely correlated with disease severity and suppression of NO synthesis increases with disease severity in Tanzanian children cohort. Further, investigators also strikingly observed that suppression of NO-mediated protective immunity after the infection may result in inadequate control of parasite replication and contributed to progression of clinical malaria in children. This very observation aptly supports our findings and are mechanistically indicative of very relevant observation in adult population that non-suppression i.e. increased expression of NO synthesis is a likely contributory factor for both mild or uncomplicated malaria and upregulated activity of NOS. Thus, elucidating the genetic factors provide a significant contribution to the variability observed in malaria; will be crucial for understanding the genetic epidemiology, malaria pathology and association studies moving towards a genome–wide era [80]. Regardless of exact functional relevance, existing evidences and based on our findings, we hypothesize that the relevant role of transition polymorphism iNOS-954G→C and −1173C→T are associated with circulating levels of nitric oxide and increasing the risk of disease susceptibility or progression in malaria infection but do not play significant role in clinical severity. Our results of iNOS polymorphism with NO level, evaluation of redox components and functional genomics during plasmodium infection suggest that evaluation of nitric oxide level and its polymorphism may be considered as a reliable molecular and biochemical marker, possess promising rational for diagnostic potential and chemotherapeutic interventions in clinical malaria. Genetic variation in iNOS promoter region is of biological significance and may play pivotal role in host defense mechanisms against malaria pathology by enhancing anti-oxidants enzymes and stimulating the infection induced biochemical cascade against plasmodium pathology. In the present study, we have applied a comprehensive and systematic genotyping based case-control research strategy to characterize common genetic variation in NOS, a gene that encodes a protein that plays diverse roles, from phase II drug metabolism to the regulation of apoptosis. Knowledge of common iNOS SNPs and haplotypes, as well as understanding of their functional implications; contribute both to mechanistic and epidemiologic studies of the involvement of NO in malarial pathogenesis as well as individual variation in response to anti-malarial drug therapy.

5. Conclusion

Irrespective of whether therapies directed against NOS will be useful in the management of clinical malaria, study of the effects of selective activation and evaluation of nitric oxide during natural infection will provide unique and valuable insights into the understanding of biochemical orchestration and patho-biology of malaria, since our current understanding of these mechanisms is limited. As the findings are novel and well aware of the limited number of subjects, deserves to be investigated in a large study group. In conclusion, our data provide link between the clinically acquired differential biochemical orchestration, infection and polymorphism through complex interplay of oxidant-antioxidant cascade in population exposed to vivax and falciparum malaria and could be contributing in the development of recombinant NO based anti-malarial drug targets. However, the reliability of nitric oxide and their polymorphisms, as a sensitive biochemical, genetic and diagnostic marker in clinical usefulness awaits further well conducted clinical investigations as to permit interventions in order to control and diagnose malaria infection.

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Competing interests

The authors have declared that no competing interests exist.

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Author contributions statement

Conceived and designed the experiments: MS, AKS. MR. Performed the experiments: AK, KPS, PB, AK, MS. SA. Analyzed the data: MS, KPS,

AKS, MR. Designed the clinical studies and collected samples: MS, AKS, MR. Contributed reagents/materials/analysis tools: NA, OPS, BKG, NK, MPS. Wrote the paper: MS, KPS.

Ethics statement and subject consent

All human blood samples used in this study were collected after obtaining consent from the study participants under protocol 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. The protocol was approved from IEC, VBU having memo no. VBU/R/885/2012 dated 05-06-2012.

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