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## ISID Research Grant Report

# *Investigation on the Role of Nitric Oxide Production and Single Nucleotide Diversity during Malaria Anaemia for Protective and Diagnostic Potential among Malaria Endemic Population of Jharkhand*

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## 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.

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 logically paves the way for alternative domain i.e. descriptive genetic epidemiology; which opens 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. 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 [1, 2].

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 [3-5], 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 [3]. 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 [6], whereas, others opine that nitric oxide has a protective role [7]. Production of nitric oxide is regulated through the enzymatic induction of. nitric oxide synthase coded by 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 [8] and other infection [9]. 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 [9,10]. Further, the role of iNOS polymorphisms may vary with endemic regions across the globe as does the manifestation of malaria. Due to the confounding evidences [11, 12] on NOS, particularly iNOS, polymorphisms and its functional importance, a number of studies have been carried out to investigate the role of these polymorphisms in disease

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susceptibility and protection, particularly in malaria and other infectious diseases.

Thus, our objective of the investigation was to analyse the differential content of nitric oxide and other associated biochemical markers, and haematological profile of the patient's actual responses upon plasmodium infection in endemic population of Hazaribag, Jharkhand, India. Apart from this, the other objective of this investigation was to evaluate the potential association between iNOS-954G/C and -1173C/T transition polymorphisms and malaria risk in the investigated population. 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.

## Materials and Methods

### 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 is as described elsewhere by Sohail et al. [17].

### Patients and Demographic Information

A total of 210 malaria patients and those without any evidence of parasites on microscopic examination i.e. healthy persons, of either sex, who attended to the local Malaria Counter at Sadar Hospital, Hazaribag, Jharkhand and were referred by general physicians of the hospital and other parts of the districts, between August 2012 to March, 2013, 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.

### Sample Collection and Clinical Investigation of Infected Subject

Peripheral venous blood (3–5 ml) was collected from all the patients before administration of antimalarial 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. 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.

### Measurement of Nitric Oxide Content

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 QuantiChromTM 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.

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

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-954 G→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. and Kun et al. [16, 18], respectively.

**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. The protocol was approved from IEC, VBU having memo no. VBU/R/885/2012 dated 05-06-2012.

**Calculation of 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 (r2) 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).

**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).

## Results and Discussion

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

We observed elevated nitric oxide content in overall malaria infected subjects as compared to healthy subjects; 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. We also analysed the gender specific concentration of nitric oxide in malarial subjects. 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 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. Interestingly, we found marginally lower concentration of nitric oxide in the highest body temperature group (100-103°F) as compared to the moderate body temperature group (99°F) and differences in the content were significant.

**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

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and p=0.0001) of body temperature with nitric oxide but weakly correlated (r2=0.003 and r2=0.04 respectively) both in malarial and healthy subjects. However, a tendency towards a marginal decrease in concentration with increase in axillary temperature was observed both in infected and healthy subjects.

**Distribution of iNOS -954G/C and -1173C/T genotype in malarial patients**

The distribution of all genotypes among controls was compatible with Hardy-Weinberg equilibrium ($\chi^2$: P=0.004 and P=0.05 for -954G/C in vivax and falciparum; P=0.04 and P=0.04 for -1173C/T in vivax and falciparum, respectively). The differences in iNOS 954G/C and -1173C/T genotype distribution among P. vivax and P. falciparum cases and controls were significantly represented. Similarly, iNOS-954 C and -1173 T allele differed significantly between cases and controls in both P. vivax (P=0.04 and P=0.04, respectively) and P. falciparum (P=0.02 and P=0.02, respectively).

**Association between iNOS Genotypes and Risk of Malaria**

Using the iNOS -954 GG and iNOS -1173 CC as the reference group, we observed that a statistically significant increased risk of malaria was associated with the -954 and -1173 combined (GC+CC) and (CT+TT) genotypes in both P. vivax and P. falciparum; respectively. 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 than among the 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 than among the P. falciparum; TT (adjusted OR=1.63; 95% CI=0.93-1.76), followed by 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).

**Association between Serum Nitric Oxide Concentration and iNOS Polymorphism in Malaria**

We observed that iNOS-954 genotypes (GG, GC and CC) group has significantly higher serum NO activity in case of vivax, falciparum and overall plasmodium infection and 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. Although, the NO content was also elevated in case of GC genotype but the difference was non-significant as compared to healthy subjects.

In regard to iNOS-1173 transition polymorphism, our observations showed that compared with healthy subjects, the iNOS-1173 genotypes (CC, and CT group) had significantly higher serum NO activity in case of vivax infection (P=0.0001, and P=0.009 for GG, and GC, 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.

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**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, r2 = 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).

## Conclusions

We found 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. 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 [13, 15]. 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 [18]. Similar to our observation regarding absence of association in terms of protective role of NOS2 promoter polymorphism; Levesque et al. [19] also did not observed consistency in protective association in the investigated population and opined that findings 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. The extent of these regional disparities of non-association is not surprising since the calculations of nucleotide diversity (ϖ), Watterson's estimate of the mutation parameter and Tajama's D are dependent on the number of low frequency SNPs identified using a given search strategy for SNP discovery. 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 [14, 19]. Since, geographic variation and susceptibility to malaria and disease phenotype may be due 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 [19, 20].

Regardless of exact functional relevance, existing evidences and based on our findings, we hypothesize that the relevant role of transition polymorphisms iNOS-954 G Ȼ and -1173 C Ŧ are associated with circulating levels of nitric oxide; increasing the risk of disease susceptibility or progression of malaria infection but do not play significant role in clinical severity. Moreover, our results expand the previous observations and are consistent with previous studies reporting the presence of association between the NOS promoter polymorphism and human malaria [19, 9].

In view of our present observations on iNOS polymorphism with NO level and other clinical parameters of plasmodium infection, we suggest that evaluation of nitric oxide level and its polymorphism may be considered to be a reliable molecular and biochemical marker,

which possess promising rational for diagnostic potential and chemotherapeutic interventions in clinical malaria. Further, our observation must be interpreted within the context of its limitations and strengths; as the findings are novel and well aware of the small number of subjects, it deserves to be investigated in a large study group.

In conclusion, this data provide link between the clinically acquired genetic adaptability, infection and polymorphism through complex interplay of nitric oxide signaling cascade in population exposed to vivax and falciparum malaria. Understanding the functional implications, genetic variability and physiological orchestration of NOS gene and its product NO is inevitable in view of their shared mechanistic pathways in regulating metabolic physiology and their diverse roles, from phase II drug metabolism to the regulation of apoptosis and modulation of vasomotor instability, oxidant stress, inflammation, endothelial adhesion molecule expression, activation of tissue factor, and platelet aggregation during malarial infection. Knowledge of common iNOS SNPs and haplotypes, as well as understanding of their polymorphic variability, risk association and genetic modulation in endemic population will contribute in improvisation of region specific epidemiological interventions as well as facilitate the underlying mechanistic elucidation of pathological cascade. 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 to permit interventions in order to control and diagnose malaria infection.

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