Nitric oxide generation in children with malaria
and the NOS2G-954C promoter polymorphism

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Running head: Nitric oxide and malaria

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Abstract

Previous epidemiological studies have demonstrated a protective association between the NOS2G-954C (NOS2<sub>Lambaréné</sub>) polymorphism in inducible nitric oxide (NO) synthase and severe malaria. The polymorphism is commoner in children with uncomplicated compared with severe malaria. We now show that the likely mechanism for such protection is increased flux of nitrogen from arginine to NO during episodes of malaria. Forty-seven boys with uncomplicated malaria received an infusion of $^{15}$N-arginine to measure directly whole body <i>in vivo</i> NO production. The NOS2G-954C genotype previously associated with reduced risk of severe malaria in Gabon was also assessed. Evaluable data were obtained from 40, of whom 6 were NOS2G-954C heterozygotes. Heterozygotes had higher urinary $^{15}$N nitrate enrichments, $2.3 \pm 0.6$ versus $1.4 \pm 0.5$ atoms percent excess ($p=0.001$) and higher ratios of $^{15}$N between urine nitrate and plasma arginine ($87 \pm 11$ versus $57 \pm 18 \%$, $p=0.001$) consistent with accelerated NO production. We also derived total NO production rates, combining data with total urine production rate and nitrate concentration; these showed no difference by genotype ($0.62 \pm 0.36$, $n=6$ versus $0.83 \pm 0.50$ umol/kg.h, $n=16$; $p=0.36$), but data were confounded by very high variability in measurements of urine output and nitrate concentrations. This study supports the idea that NOS2 genotype protects against severe malaria by increasing NO production during episodes of uncomplicated malaria.

Keywords

Malaria, nitric oxide, nitric oxide synthase, polymorphism
Introduction

The nitric oxide radical (NO) modulates key physiological processes and antimicrobial defenses. NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). The inducible form of the enzyme, iNOS or NOS2 (NOS1 and NOS3 being constitutive enzymes) is found in many cells and tissues that are important in orchestrating immunoregulatory processes. Examples include macrophages, where NO has a role in parasite killing (29) and endothelial cells where vascular tone and adhesion-receptor expression play a critical role in immune responses (29). NOS2 can elicit high levels of NO production and its expression is largely controlled by pro-inflammatory cytokines through transcriptional mechanisms (10).

Several years ago, we noted that a polymorphism in the NOS2 gene, NOS2G-954C (NOS2Lambaréné), was distributed non-randomly in children with severe and uncomplicated malaria in Gabon, being more frequent in children with uncomplicated disease (18). The NOS2G-954C polymorphism was also associated with significant delay in time to re-infection with malaria, when compared with children who did not carry it (17). Extending these studies to other regions confirmed that this polymorphism is found in other malaria-endemic regions, such as Tanzania (19), is present in Americans of African origin (19), but is very rare in SE Asia (3)(9), and absent in white Americans (19) and Germans (18). Heterozygosity for the NOS2 promoter G954C polymorphism, but not C1173T, was associated with a significantly lower incidence of malaria compared with carriage of the wild-type allele in children in Uganda (IRR = 0.69, P = 0.05) (23). However, not all studies have found a protective association from this genotype. In Tanzanian children no consistent associations were found between several NOS2 promoter haplotypes and malaria severity/anemia, NOS2 expression or plasma/urine [nitrate + nitrite] (NOx) concentrations (19, 20), although in one subgroup the G954C
haplotype was associated with protection from clinical malaria (20).

What is the mechanism for such putative protection? Malaria induces NOS expression in peripheral blood mononuclear cells (PBMC) of children with malaria, an effect promoted by parasite-generated haemozoin (15). G954C heterozygosity is associated with higher plasma nitrate levels in young children with malaria (<24 months) (7) and with elevated unstimulated NOS enzyme activity in PBMC (17). Similarly, the C1173T polymorphism also appears to protect by increased NO synthesis as reflected by increased fasting urine and plasma NO metabolite concentrations in Tanzanian children (13). Taken together, these data, suggest that specific genotypes promote enhanced NO responses which protect against severe malaria (7, 13, 15, 29). but the direct in vivo physiological link has not yet been conclusively demonstrated.

One of the difficulties establishing the effect of a specific genotype on NO metabolism is that surrogate measures of NO production such as plasma and urine nitrate/nitrite (NOx) concentrations are highly variable, being profoundly influenced by dietary nitrate intake, urinary output and changes in renal function. Even after controlling for dietary influences and correcting with creatinine concentrations, NOx levels do not provide wholly reliable estimates of NO production. These methodological limitations can be avoided by using isotopically-labelled arginine, which distinguishes between nitrate derived from NO production and that arising from other sources (22).

We took this latter approach, using 15N-labelled arginine to measure in vivo rates of NO production in children with uncomplicated malaria. We then applied the principle of Mendelian deconfounding to test the original hypothesis that increased nitric oxide production protects against severe malaria (27), by relating results of in vivo NO
production in patients with uncomplicated malaria to the presence of the NOS2G-954C gene polymorphism for NOS2. Tests of this hypothesis are particularly relevant as interventional studies designed to increase NO production in malaria are being implemented (31).
Methods

Subjects and Clinical Assessment

Male children with uncomplicated malaria aged between 3-10 years attending the Albert Schweitzer Hospital, Lambaréné, Gabon were recruited to the study. Only male subjects were included to facilitate collection of urine samples. Uncomplicated malaria was defined by fever (rectal temperature >38°C) and parasitemia (the presence of 20,000-200,000 parasites/μl of asexual stages of *Plasmodium falciparum* in thick or thin blood films), in the absence of clinical or biochemical evidence of severe malaria (such as repeated vomiting, convulsions, coma, severe anemia (PCV<20%), hyperlactatemia (≥5mmol/l) or hypoglycemia (≤2.2mmol/l)). Alternative diagnoses were excluded clinically. Children were admitted and managed for malaria according to standard protocols as described elsewhere (24), receiving a single oral dose of sulfadoxine/pyrimethamine as therapy. All study procedures were approved by the Ethics committees of the International Foundation of the Albert Schweitzer Hospital and the Gabonese Ministry of Health and informed consent was obtained from all parents.

Clinical Protocol

In order to investigate NO metabolism during an episode of malaria, we used *in vivo* labeling with guanadino-¹⁵N₂-arginine as previously described (22). Since the guanadino nitrogens of arginine are the substrate for NO production, the rate of NO production can be derived from the rate of appearance of ¹⁵N-nitrate in urine. Subjects received L-[guanadino-¹⁵N₂]arginine (Cambridge Isotopes Laboratory, MA) as an intravenous infusion at a rate of 2 μmol/kg/h over 12 hours after a priming dose (2 μmol/kg) to achieve rapidly steady-state levels of enrichment in plasma arginine. Plasma samples (0.4 ml) were taken for measurement of arginine enrichment at base-line and at approximately hourly time-points from 6 hours to the end of the study. At the start of the
infusion 14 µg/kg of ^15N-sodium nitrate was given as an oral solution to prime the urinary nitrate pool with ^15N; this avoids long equilibration times before plateau measurements of urinary ^15N can be made (22). A urine sample was collected at the beginning of the study, when subjects were encouraged to void urine. Subjects were encouraged to void again at 6 hours, 8 hours and the end of the infusion and all urine volumes were measured and aliquots (≥5 ml) retained for ^15N-nitrate analysis. If boys did not void after 12 hours, the infusion was continued until voiding. No dietary restrictions were placed on the children during the study, as ^15N-nitrate measurements should be independent of other nitrate fluxes; intake was not formally measured but all children were unwell and most ate nothing or only very little during the study. The normal Gabonese diet is low in meat, consisting mainly of rice or bread and vegetables.

**Estimation of NO Production *in vivo***

Four parameters were derived for calculation of NO production rate:

1. **Plasma arginine enrichment.** Plasma arginine enrichment was measured by gas chromatography-mass spectrometry (GC/MS) of the n-butyl-heptafluorobutyryl derivative of arginine (DB5-MS column, Agilent 5973/6980 GCMS, Agilent, Bracknell, UK) as previously described (22).

2. **Urinary nitrate enrichment.** Urinary nitrate enrichment and concentration were measured by a novel method in which nitrate is trapped as nitromesitylene by reaction with mesitylene in the presence of trifluoroacetic anhydride (14). Samples were analyzed by GC/MS (Agilent 5973/6980 using a DB5-MS column) monitoring ions m/z 165 and 166 corresponding to the unlabelled and labeled moieties of nitromesitylene, respectively.

3. **Urinary nitrate concentration.** Nitrate concentration was measured in aliquots of urine by “spiking” aliquots of urine with known amounts of ^15N-sodium nitrate as
internal standard and comparing the enrichment of unspiked and spiked samples by GCMS, as described above.

(4) **Total urinary nitrate production rate.** This was estimated from the product of urinary nitrate concentration and timed urine volumes. Using these parameters, NO production rate was calculated from the equation:

\[
\text{Steady state NO production rate} = \frac{\text{Rate of Excretion of } ^{15}\text{N nitrate}}{\text{Plasma Arginine } ^{15}\text{N Enrichment}}
\]

Equation (1)

Where, \( \text{Rate of Excretion of } ^{15}\text{N nitrate} = \text{Total nitrate concentration in urine} \times \text{Urine production rate} \times ^{15}\text{N enrichment of nitrate} \)

Equation (2)

Results were expressed in absolute terms (μmol/h), and per unit total body weight (μmol/h.kg).

Previous studies have suggested that recovery of labeled nitrate may be incomplete(28), so measured values may be underestimates in absolute terms; our data is shown without applying a compensatory correction factor to avoid potential bias. NO production data were analyzed blinded with respect to NOS promoter genotype and data rejected if steady state was not reached, if critical data were missing or if wide variance made plateau estimation impossible.

**DNA analysis**

DNA was extracted from circulating leucocytes and analyzed for status of promoter polymorphisms using published PCR protocols after extraction of genomic DNA with the QIAamp blood kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was carried out on a Rapid Cycler (Idaho Technologies, Idaho) in 15-μL reactions using the
following conditions: an initial 15-s denaturation step at 94°C, followed by 40 cycles between 94°C (no dwell-time), 60°C (no dwell-time), and 72°C (30s dwell-time). Primers were:

NOS-F (5'-CATATGTATGGGAATACTGTATTTCAG-3'), and
NOS-R (5'-TCTGAACTAGTCACTTGAGG-3').

The generated fragments were sequenced using BigDye chemistry (Applied Biosystems, Foster City, CA) followed by subsequent analysis on an ABI Genetic Analyzer 3100 (Applied Biosystems, Foster City, CA).

**Statistical analysis and power estimation**

Sample size for the study was determined assuming (i) the frequency for heterozygosity for the NOS2 polymorphism in Gabon is 30%, as previously found (18), and (ii) that the coefficient of variation for estimates of weight-normalized NO production, including analytic and inter-individual variance, would be ≤29%, as found in previous studies using the same approach (22). To achieve an 80% power to detect a 33% difference between heterozygotes and wild-type subjects would require a total sample size of ≥27 cases of mild malaria. However, to assure recruitment of at least 9 heterozygotes, we set out to recruit 40-50 total cases. Normality was tested by the D'Agostino and Pearson test and, if acceptable, differences between groups were tested by 2-tailed Student’s *t* test (*Prism*, GraphPad Software Inc., La Jolla, CA).
Results

Forty seven boys aged between 34-128 months entered the study. Demographic and clinical variables for the 40 subjects included are summarized in Table 1 (data from 7 children were excluded from further analysis because of missing samples or incomplete data). Genotyping revealed 33 subjects had the wild-type promoter sequence (NOS2G-954) for NOS2, 6 were NOS2G-954C heterozygotes and one was homozygous for this polymorphism. Clinical characteristics and level of parasitemia were similar for wild-type homozygotes and heterozygotes.

When $^{15}$N enrichments were assessed, plasma arginine enrichment reached a plateau in most subjects at about 2-3 atoms percent excess (APE) (mean 2.39 APE) after about 6 hours; a typical plot is shown in Figure 1. This enrichment equates to an average arginine flux through the plasma pool of ~84 $\mu$mol/hr.kg total body weight, in a similar range, but about 50% higher than values obtained previously in healthy adults, 56 $\mu$mol/hr.kg (recalculated from (22) for total weight). Urinary nitrate enrichment reached plateau in most subjects at ~1.5 APE. In two (wild-type) subjects isotopic plateau estimation was not possible and in a further four (all wild-type), only usable nitrate or arginine enrichment data were available, but not both.

When isotopic enrichments were compared, we found that urinary nitrate $^{15}$N enrichments were significantly higher in heterozygotes for NOS2G-954C than in wild-type subjects, 2.27 ± 0.63 versus 1.37 ± 0.51 APE respectively ($p=0.001$ by Student’s $t$-test; Table 2, Figure 2). Furthermore, the ratio of urinary nitrate enrichment to plasma arginine enrichment, which gives an index of the proportion of urinary nitrate arising from NO production, was also significantly higher in heterozygotes than in control subjects: 87 ± 11 % versus 57 ± 18 % ($p=0.001$ by Student’s $t$-test; Table 2, Figure 2). The
value from the single homozygote (for NOS2C-954) is given in the Table but no
comparisons were made with other groups in view of the fact that this was a sole
individual. Thus heterozygosity for NOS2G-954C appears to be characterized by higher
rates of conversion of arginine to nitrate in children with uncomplicated malaria.

We also attempted to derive data on total nitrate excretion (reflecting constitutive as well
as inducible NO production) but data were confounded by the difficulty collecting
complete and accurate timed urine collections in sick young boys in field clinical settings
(we wished to avoid clinically unnecessary catheterization); refusal or inability to pass
urine, partial voiding and incomplete urine collections were common. As a consequence,
we found very wide inter-individual variation in calculated total nitrate production rates,
ranging by a factor of almost ten (minimum 0.3, maximum 2.5 µmol/h.kg, Table 2,
Figure 2), whether normalized for body weight or body surface area. Combining nitrate
production rate data with enrichment data yielded total NO production rates with
similarly wide variance resulting in data which overlapped between groups: 9.9 ± 4.9
µmol/h in heterozygotes versus 16.0 ± 11.9 µmol/h in wild-type subjects (p = 0.22, Mann
Whitney U test) and coefficients of variation of >50% (cf. values of <30% from previous
studies (22) used for power calculations). Total NO production rates, normalized for
body weight, were 0.83 ± 0.50 µmol/h.kg in wild-type and 0.62 ± 0.36 µmol/h.kg in
heterozygotes. Normative data are not available for children but these values are 3-4-fold
higher than those seen in healthy adults 0.23 ± 0.04 µmol/h.kg (recalculated for total
body weight from (22); n=8; p < 0.001 and p < 0.05 respectively). Normalizing for body
surface area rather than weight yielded similar conclusions (data not shown).
Discussion

Some studies have suggested a protective role against malaria for the NOS2G-954C polymorphism (NOS2Lambaréné) in African children (17). We now provide evidence that the mechanism for this protection is enhanced synthesis of nitric oxide from arginine. Using in vivo labeling studies, we find that during an episode of uncomplicated malaria heterozygotes for the NOS2G-954C polymorphism have higher rates of conversion of arginine to NO (by a mean of approximately 66%) when compared with those without the polymorphism. (We excluded severe cases to avoid a potential selection bias, as we hypothesized that progression from uncomplicated to severe disease would itself be associated with alterations in NO production.) This study provides a mechanistic pathway explaining how the innate NO defense pathway may limit the impact of P. falciparum infection, consistent with previous work from Gabon and Uganda showing reduced episodes of clinical malaria in those carrying the NOS2G-954C polymorphism (17, 23).

There are several mechanisms by which NO may exert its protective effects in malaria. First, NO is directly parasiticidal in vitro (2, 25). Previous work has shown inconsistent relationships between parasitemia in vivo and indirect measures of NO production such as plasma nitrogen oxides (7), although elevations in NO₅ are sometimes inversely correlated with peripheral parasitemia (1, 16). In this study, there was no relationship between admission parasitemia and measures of NO synthesis. Second, increased NO may modulate many aspects of endothelial cell function in ways that protect against development of severe disease (30) (31). For example, NO counteracts the up-regulatory effects of pro-inflammatory stimuli on expression of endothelial cell adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM) (8, 26). This would be predicted to decrease the risk of complications resulting
from microvascular obstruction in severe syndromes of malaria by reducing cytoadhesion of parasitized erythrocytes. Consistent with this suggestion, low NO bioavailability is associated with poorer outcome in an animal model of cerebral malaria (12). A possible mechanistic role for NO in the coma of human cerebral malaria has been reviewed recently (6).

In this study we also attempted to evaluate differences in whole body NO production rates between heterozygotes and wild type subjects but found no significant difference. We believe this is because whole body NO production estimates were confounded by uncertainty in the true values for urine production rate and nitrate concentration. Furthermore, it appears that not all nitrate produced systemically will appear in the urine (28). Comparing serial samples (data not shown) we found very high intra-individual variability in urine production rates and in urinary nitrate measurements (urine dilution sometimes changing dramatically with rehydration after admission). Such observations highlight the difficulties inherent in the use of NO\textsubscript{x} data alone to derive NO production, particularly in clinical or field settings and explains some of the variability seen in other published studies. The use of \textsuperscript{15}N-arginine as a specific tracer for NO synthesis allowed us to circumvent such difficulties by making direct comparisons between \textsuperscript{15}N enrichment in plasma arginine and urinary nitrate. An alternative model mathematically consistent with our findings is that children with the wild-type promoter had a difference in \textsuperscript{15}N nitrate enrichment but not total NO production because they excreted larger amounts of unlabelled nitrate. It is hard to conceive a physiological mechanism by which this might occur unless dietary nitrate intake were very biased between the two groups; although we did not control dietary intake there is no reason to suppose this was the case. This study was limited by a number of subjects whose data could not be used; longer infusion times may have improved the proportion achieving
plateau values but would have been logistically more difficult. When compared with historical control healthy adults, our data are consistent with accelerated NO production in children with malaria, although the relative contributions of age and disease cannot be separated.

Why is the geographical distribution of the NOS2G-954C polymorphism restricted to areas that have malaria? This observation would be consistent with deleterious consequences of the heterozygous state in non-malaria-affected populations, although alternative explanations warrant further investigation. An analogy may be drawn here with sickle cell trait, although there are no data to support the notion that a homozygous state for the polymorphism (NOS2G-954C) is deleterious to the carrier. This polymorphism is found in populations of African origin but not found in Caucasians nor in almost all Asians (3). The possibility of linkage disequilibrium between NOS2 haplotypes must also be considered (4, 19). Other polymorphisms of NOS2 may also contribute to variation in NO synthesis, including single nucleotide substitutions at positions -1173 (C-1173T) (13), and -1659 (C-1659T) (4) as well as a microsatellite repeat (CCTTTn) 2.5 kb upstream from the NOS2 transcription start site (5). Although such genotypes have been associated with protection from malaria, they were not included in our primary hypothesis for testing; we chose to focus on NOS2G-954C in this study because of its high local prevalence, but the possibility of linkage disequilibrium between NOS2 haplotypes must also be considered (4, 19).

Despite best-available parasiticidal therapy, mortality from malaria is still significant, especially in children, emphasizing the need for better adjunctive therapies. Of the mortality associated with childhood malaria, a significant proportion is related to cerebral disease. Endothelial adhesion of parasitized erythrocytes is a contributor to
pathology and modifying NO metabolism is therefore one potential avenue towards increasing survival. Proposals to consider interventions that aim to do this should include consideration of the effects of host genotype on whole body NO production.

One proposed strategy is to administer arginine directly on the basis that arginine availability may become rate-limiting for NO production during an episode of malaria (21)(12). In support of such an approach, some data suggests that arginine flux may be accelerated in infected individuals (32). Consistent with this, our values for arginine flux were 50% higher than in healthy adults studied using the same methodology, although the relative contributions of age and disease cannot be distinguished. This accelerated flux is probably not solely related to increased NO production as only about 5% of plasma arginine flux is directed towards NO synthesis in the healthy state (22). Arginine infusion appears to be safe (32) but efficacy data are awaited.

**Perspectives and Significance**

This study has extended our previous epidemiological observations by providing a mechanistic link between the NOS2G-954C polymorphism and *in vivo* NO physiology. This study also has methodological implications; the use of $^{15}$N-arginine as a tracer, combined with a novel assay for $^{15}$N in nitrate (14), allowed us to identify changes in NO flux which probably would not have been apparent from nitrate measurements alone. This study also supports the further exploration of NO manipulation as adjunctive therapy in the treatment of malaria.
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References


Figure Legends

**Figure 1. Example of nitrate fluxes and $^{15}$N enrichment**

Data are from one subject. (a) Total nitrate production rate (filled squares) as the product of urine volume (filled circles), and urinary nitrate concentration (open squares), which tends to rise as urine flow rate falls. (b) $^{15}$N enrichments in plasma arginine (filled diamonds) and urinary nitrate (open circles) during primed constant infusion of $^{15}$N arginine starting at time zero. Data are expressed relative to the baseline value at time zero.

**Figure 2. Effect of NOS2G-954C promoter polymorphism on products of NO synthesis**

(a) Enrichment of $^{15}$N in urinary nitrate in atoms percent excess (APE), (b) ratio of urinary nitrate to plasma arginine, and (c) total NO production, in boys receiving $^{15}$N arginine. Results are displayed according to NOS2 genotype: WT, wild-type, Hetero, heterozygous. Differences between wild-type and heterozygotes are significant in (a) and (b), p<0.001, by Student’s t-test, but not-significant in (c).
Figure 1. Example of nitrate fluxes and $^{15}$N enrichment
Figure 2. Effect of NOS2G-954C promotor polymorphism on products of NO synthesis

- Urinary nitrate ¹⁵N enrichment (APE)
- ¹⁵N Nitrate / Arginine ratio (%)
- NO production (umol/kg.h)
Table 1. Demographic characteristics

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No significant differences were found in any of the parameters shown between wild-type and heterozygotes by student’s t-test (2-tailed) or Mann Whitney U test for non-parametric comparisons.
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* P<0.01 by Student’s t-test, two tailed. † In 4 subjects only arginine or only nitrate data were available.