An investigation of mechanisms proposed to mediate anti-toxic immunity to malaria

by

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To Joseph Slagi, Ferdinand Baighi, Peter Marum and all the other villagers of Genyem, Haven and Midiba.

This research is for you.
Declaration

I hereby declare that the work herein, now submitted as a thesis for the degree of Doctor of Philosophy of the Northern Territory University, is the result of my own investigations, and all references to ideas and work of other researchers have been specifically acknowledged. I hereby certify that the work embodied in this thesis has not already been accepted in substance for any degree, and is not being currently submitted in candidature for any other degree.

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Abstract

It is not known how individuals chronically exposed to malaria transmission have adapted to enable a life relatively free of malaria symptoms despite the ongoing persistence and replication of *Plasmodium* species in their bloodstreams for much of the time. It has been supposed that a combination of innately determined and acquired factors contribute to the maintenance of apparently good health in the face of chronic parasitisation by inhibiting the inflammatory events triggered by parasite toxins. Candidate mechanisms proposed to mediate this state of malarial “tolerance” include enhanced production of the biological messenger nitric oxide (NO; which may down-regulate events such as the release of endogenous pyrogens) and neutralising antibodies against the proposed *P. falciparum* toxin, glycosylphosphatidylinositol (GPI). Prior to this study, it was assumed that each mechanism was either boosted (NO) or induced (anti-GPI antibodies) by malaria infection in proportion to the level of parasitemia, which in highly endemic regions is commonly highest in early childhood and lowest in adulthood.

As much of the evidence that informed these two hypotheses was derived from *in vitro* and animal studies, the purpose of this study was to test each of them under field conditions in populations chronically exposed to intense malaria transmission. The first project was a small cross-sectional pilot study involving adults in Papua province (formerly Irian Jaya) and the second enrolled children and adults from Madang, Papua New Guinea (PNG) who were given treatment to eradicate parasitemia and then followed longitudinally to evaluate its effect. Total systemic NO production was estimated along with cellular production by peripheral blood mononuclear cells (PBMCs), thought to be most receptive to induction by infection and high output NO production. Anti-GPI immunoglobulin (Ig)M, IgG and IgG subclass antibodies were also determined cross-sectionally and longitudinally.

The results of the study presented in this thesis argue against both of these hypotheses. Although systemic NO production and PBMC NO synthase (NOS) activity were significantly higher at both study sites than in Darwin controls, there was no evidence that NO production was age-dependent and no absolute requirement
for malaria parasitemia to induce NO was demonstrated. The disassociation of systemic and PBMC NO production suggests that other cellular sources may be at least as contributory as PBMCs to the overall high basal levels of NO. Genetic polymorphisms thought to influence disease outcome through altered NO production in other geographical locations were either not found or found not to influence NO production/NOS activity in the PNG study subjects. This suggests that linkage disequilibrium with other critical polymorphisms may have explained the results elsewhere, that other polymorphisms are more important in differential NO production in PNG, and/or that more care needs to be taken interpreting the results of disease association studies. Anti-GPI antibody production was shown to be age-dependent, with increasing prevalence, abundance and persistence with increasing age. IgG predominated over IgM and was short-lived, which was shown to be most likely due to a skewing of anti-GPI IgG subclass production to the more transient IgG₃ over IgG₁. As very few young children with high parasitemias produced antibodies, they are very unlikely to be the sole mediator of tolerance, if at all.

This study focuses attention on other potential mechanisms for mediating tolerance, the understanding of which could inform strategies aimed at reducing death from malaria, as well as the burden of disease. Conversely, the results of this study can potentially inform further investigation of other proposed anti-toxic roles for NO and anti-GPI antibodies in retarding disease severity.
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**Declaration of author’s contribution**

This thesis is substantially my own work and was performed under the guidance of my supervisor Nick Anstey. I implemented and led the field work at both study sites and performed the following laboratory procedures and assays: processing of specimens and separation of PBMCs in PNG (Chapter 9.1); manual WCC estimation (Chapter 8.6.2); nitrite and nitrate quantification in urine and plasma (Chapter 9.2); whole genome amplification PCR (Chapter 9.5); genotyping of IL-12 polymorphisms for the PNG study (Chapter 9.6); genotyping of the G-954C polymorphism (Chapter 9.7); anti-GPI IgG and IgM ELISA (Chapter 9.9); anti-GPI IgG subclass ELISA (Chapter 9.10); IL-18 ELISA (Chapter 11.2.3) and the abandoned prostaglandin E2 ELISA (Chapter 9.4). I also performed all of the statistical analyses presented in this thesis with occasional advice from Zhiqiang Wang. Recruitment of Jayapura controls (Chapter 11.2.2.2) was conducted by Helena Maniboey for a related study under the direction of Nicholas Anstey. Recruitment of Tanzanian subjects and controls, and performance of the related assays, were previously conducted by Nicholas Anstey (Chapter 13.2).

I wrote all of the papers and abstracts on which I was first author. I contributed to the chapter in *Malaria Methods and Protocols* on which I was second author by writing those sections which I had modified from previously published methods. I performed all of the statistical analyses, wrote all of the results, part of the methods and contributed substantially to the discussion (with a lesser contribution to the introduction) of the paper in *Genes and Immunity* on which I was second author. The section of the thesis pertaining to that work has been extensively rewritten and expanded such that it reflects my own interpretations and not those of the co-authors (Chapter 13.2).

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Publications

Refereed journals


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**Book chapter**


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**Boutlis CS**, Anstey NM. Tolerance of malaria parasitemia in malaria endemic population does not appear to be mediated by nitric oxide or antibodies to *Plasmodium falciparum* glycosylphosphatidylinositol as previously proposed. Proceedings of the Annual Scientific Meeting of the Australasian Society for Infectious Diseases, Canberra, 2003.


**Boutlis CS**, Tjitra E, Utomo G, Misukonis MA, Suprianto S, Granger DL, Weinberg JB, Anstey NM. Markedly elevated nitric oxide (NO) production peripheral blood mononuclear cell (PBMC) nitric oxide synthase (NOS2) expression in asymptomatic malaria-exposed adults in rural Indonesia; with highest NO production in those with

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### Abbreviations

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<tbody>
<tr>
<td>ABTS</td>
<td>2,2’-azino-di-(3-ethyl-benzthiazoline-6-sulfonate)</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BSA</td>
<td>body surface area</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>CM</td>
<td>cerebral malaria</td>
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<tr>
<td>Cr</td>
<td>creatinine</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
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<td>circulating sequestered</td>
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<td>CS</td>
<td>circumsporozoite</td>
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<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>DHFR</td>
<td>dihydrofolate reductase</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>FENOx</td>
<td>fractional excretion NOx</td>
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<tr>
<td>GDP</td>
<td>gross domestic product</td>
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<tr>
<td>GFR</td>
<td>glomerulofiltration rate</td>
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<tr>
<td>GlcN</td>
<td>glucosamine</td>
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<tr>
<td>GM</td>
<td>geometric mean</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
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<tr>
<td>HC</td>
<td>healthy control</td>
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<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HREC</td>
<td>human research ethics committee</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<td>IFN</td>
<td>interferon</td>
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<td>IgE</td>
<td>immunoglobulin-E</td>
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<td>Abbreviation</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>P-EtN</td>
<td>phospho-ethanolamine</td>
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<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
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<tr>
<td>PKC</td>
<td>protein kinase-C</td>
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<tr>
<td>PNG</td>
<td>Papua New Guinea</td>
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<tr>
<td>PNGIMR</td>
<td>Papua New Guinea Institute of Medical Research</td>
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<tr>
<td>PTK</td>
<td>protein tyrosine-K</td>
</tr>
<tr>
<td>RAP</td>
<td>rhoptry-associated protein</td>
</tr>
<tr>
<td>RESA</td>
<td>ring-infected erythrocyte surface antigen</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>$T_0$</td>
<td>time-point 0 - baseline</td>
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<tr>
<td>$T_1$</td>
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<tr>
<td>$T_2$</td>
<td>time-point 2 - 2$^{nd}$ follow-up</td>
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<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>UM</td>
<td>uncomplicated malaria</td>
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<tr>
<td>UTI</td>
<td>urinary tract infection</td>
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<tr>
<td>WCC</td>
<td>white cell count</td>
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<td>WGA</td>
<td>whole genome amplification</td>
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<td>WHO</td>
<td>World Health Organization</td>
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SECTION One.
Background
Chapter 1. Introduction and scope of this thesis

Malaria is an infectious disease of immense global importance that continues to kill one child every 40 seconds [1]. Yet, paradoxically, the closer a population’s relationship with the parasite the better the individuals within that population appear to deal with it, both in terms of fewer clinical attacks and episodes of lesser severity [2]. Nowhere is this more evident than in regions of particularly high malaria endemicity, where chronic parasitisation of individuals for long periods without symptoms is the rule, rather than the exception. As the malaria parasite Plasmodium falciparum has ancient origins that may even predate human population expansion [3], this unique host-parasite relationship is likely to represent the pinnacle of adaptation by both sides. Disentangling the genetic, immune, social and geographic factors contributing to this equilibrium is therefore fundamentally important to the design of novel preventive and therapeutic strategies aimed at reducing the burden of disease.

How it is that humans have managed to maintain reasonable health in the face of ongoing replication of a potentially pyrogenic protozoan within their bloodstream has intrigued researchers for over 100 years but has not yet been resolved. Consistent with the notion that malaria induces symptoms such as fever coincident with rupture of parasites and the release of toxins in the latter half of its blood stage life cycle is the hypothesis that asymptomatic chronically parasitised humans have developed an anti-toxic immune response. This response (which can be termed “tolerance”) appears to depend on prior infection and fundamentally differs from other immune responses directed at killing off parasites in that it appears to decrease rather than increase with advancing age. It goes without saying that this tolerance is lost or overcome upon the development of clinical malaria. In addition, the severity of clinical malaria varies among individuals inferring that different levels of anti-toxic immunity may potentially influence disease outcome. Investigating the mechanisms proposed to mediate these anti-toxic immune responses is the purpose of this thesis.
Research attention has predominantly focused on the biological messenger nitric oxide (NO) and toxin-neutralising antibodies as candidate anti-toxic immune mechanisms. A large body of circumstantial evidence gathered mostly (but not exclusively) from in vitro and in vivo animal studies has accumulated to support a role for either or both of these propositions. The value of in vitro studies is that individual processes can be studied in isolation but their worth is necessarily limited by their reduction in complexity compared to the in vivo situation. In vivo studies using non-human animals with malaria parasites that do not parasitise humans can at best only suggest avenues for exploration that require confirmation in human studies. Furthermore, animal studies also lack complexity by not accounting for other factors that may influence anti-malarial immune responses (such as concomitant infection with other organisms). Therefore, the aim of this thesis is to examine NO production and anti-toxin antibodies in chronically parasitised children and adults living on the island of New Guinea in the context of exposure to perennially high malaria transmission.

The background information presented in this section of the thesis concentrates mainly on the toxic basis of malarial pathology, as well as the epidemiology and physiology underlying anti-toxic immune responses. This section is deliberately detailed so as to clearly present the nature and strength of the evidence upon which the hypotheses to be tested in this thesis are founded. For the most part, the literature review is current as of November 2002, although it should be recognised that the field studies conducted to address the research questions were formulated on the basis of evidence available through February 1999. In a few instances, the review of relevant literature is held over for discussion in the context of the data presented in the field studies. The second section describes general experimental and laboratory procedures relevant to the field studies. The third section deals with the presentation, analysis and discussion of data generated from field studies in the Papua province of Indonesia and in the Madang province of Papua New Guinea (PNG). The fourth and final section concludes the thesis with a discussion of the relevance of this work in relation to the questions it was seeking to answer and in the context of how these results could be used to further research in this field.
Chapter 2. General malariology

Malaria is the world’s deadliest parasitic disease, killing more than 1 million of the planet’s poorest children every year [4]. For more than 40% of humanity [5], surviving childhood ensures a lifetime punctuated by episodes of malarial fever that require increasingly expensive drugs to treat. In this day and age it is ironic that a single cell protozoan with 14 chromosomes containing only 23 million base pairs [6] has managed to outsmart complex multicellular organisms now known to contain 3.2 gigabases of deoxyribonucleic acid (DNA) neatly arranged into more than 30000 genes [7]. The *Plasmodium* species in question have comfortably evaded all attempts at vaccination, seen off a variety of pharmacological agents and probably infect more people today than ever before. In doing so, the parasite has come to occupy a socio-political niche such that, “where malaria prospers most, human societies have prospered least” [1]. How these events have come to pass will be outlined in the following chapter.

2.1. Life cycle

*Plasmodium falciparum, vivax, malariae* and *ovale* are only known to infect and reside within humans, being transmitted from person to person by an intermediate host in most instances or rarely, by contaminated blood. The life cycle of each species begins with mating of sexual forms (gametes) in the midgut of female *Anopheles* mosquitoes (Figure 2.1). Fertilisation of gametocytes is followed by development and replication of asexual forms within the mosquito that continues for the next week or so and ends with the migration of sporozoites to salivary glands. Sporozoites are particularly trophic for human hepatocytes, which they invade within about 45 minutes of the mosquito’s next blood meal before undergoing further replication (unbeknown to the host).
Figure 2.1. Life cycle of malaria parasites in humans
Figure reproduced with the kind permission of Ric Price.

Subsequent events are critically dependent on the nature of host immunity. In people with little or no immunity: the primary incubation period is generally completed within 5.5 (P. falciparum) to 15 (P. malariae) days and is followed by the release of thousands of merozoites from ruptured hepatocytes that rapidly attach to, and then invade erythrocytes [8]. Further asexual development followed by multiplication occurs within erythrocytes, which necessarily enlarge, distort and become less deformable. At least in the case of P. falciparum, a number of parasite encoded proteins are expressed on the red cell surface during this time which have a role in pathogenesis. Merozoites become trophozoites, which develop into multinucleated schizonts and then cause the erythrocyte to rupture after 48-72 hours, liberating approximately 20 merozoites per erythrocyte. Invasion of uninfected erythrocytes occurs rapidly, leading to a phase of logarithmic expansion in parasite densities. Fever and other symptoms begin to occur once a threshold of infection is reached that is enough to overcome the host’s innate resistance. At some stage, soon after exiting the liver in the case of P. vivax but after a delay in the case of P. falciparum, the asexual forms may develop separately into male and female gametocytes which circulate in the bloodstream available for ingestion by a female Anopheles mosquito.
In subjects with prior exposure to malaria, the *Plasmodium* life cycle may be affected by host immune responses at each stage of its development that modify both the likelihood and severity of symptoms (Chapter 4).

### 2.2. Epidemiology

The clinical epidemiology of malaria is primarily determined by the intensity of malaria transmission (which heavily influences host immunity) and species of infection, with only *P. falciparum* causing life-threatening illness. Previously malaria-naïve individuals are at highest risk of disease, but even so, the severity of untreated disease ranges from characteristic febrile paroxysms that recrudesce and relapse over long periods of time, to rapid death [9]. Somewhat paradoxically, the overall burden of disease (both mild and severe) relative to the number of infections appears lower in regions of higher endemicity (like PNG or sub-Saharan Africa), where even newborn babies enjoy a period of relative protection [10]. The age pattern and phenotype of disease is also closely correlated with the intensity and seasonality of transmission. In highly endemic areas of stable (non-seasonal) transmission, severe disease is mainly confined to early childhood and is defined by three dominant but overlapping syndromes: severe anemia (causing hypoxia); cerebral malaria (causing coma); and severe metabolic acidosis (revealed clinically as respiratory distress) [11]. Life thereafter is punctuated by episodes of uncomplicated disease that decrease in frequency with age [12,13]. The presentation of severe disease is different in areas of low or unstable malaria transmission, with manifestations such as cerebral malaria being disproportionately more common and occurring at a later age [14]. A notable exception to the general picture outlined above is the less benign behaviour of *P. falciparum* during pregnancy, which may cause severe disease and maternal deaths in both low and high transmission settings [15].

An intriguing aspect of malaria epidemiology in regions of high endemicity relates not to disease but rather to the occurrence and persistence of asymptomatic infection. This is one of the few reliable indicators of immunity (Chapter 4), and along with spleen enlargement rates, one of the best markers of malaria endemicity [16]. The point prevalence and density of asymptomatic infections generally peaks around the
same time or slightly later than that of severe malarial disease in areas of stable endemicity and has been reported to reach rates close to 90% in parts of PNG and Africa [17,18]. The ability to sustain and maintain asymptomatic infections is diminished in regions of unstable or seasonal transmission [19]. The presence and prevalence of asymptomatic infection has traditionally been defined on the basis of single microscopy readings, however, there has been increasing recognition recently of the inherent lack of sensitivity in such an approach [20]. The use of molecular techniques such as polymerase chain reaction (PCR) has enabled detection of lower parasite densities, particularly in adults [21], and has helped define the dynamics of fluctuation in parasite densities above and below the microscopic detection threshold in children [22]. In the light of these epidemiological observations, it appears that few, if any individuals ever gain the ability to live a life free of malaria.

2.3. Diagnosis

2.3.1. Clinical malaria and attributable fraction estimation

The diagnosis of severe malaria according to standardised World Health Organization (WHO) criteria is relatively straightforward [15]. Diagnosing uncomplicated clinical malaria in populations where asymptomatic infection is common and the clinical features are non-specific is far more difficult but still important for estimating the burden of disease and for determining who should receive anti-malarial treatment. The question therefore arises in sick individuals as to whether or not an observed parasitemia is causal or incidental. Using classical epidemiological methods [23], the prevalence of parasitemia in febrile and asymptomatic individuals can be used to calculate the proportion of cases in febrile individuals attributable to malaria (the “attributable fraction”). Intuitively, it is obvious that this method makes sense. For example, if the rate of parasitemia in asymptomatic individuals is 40%, and in sick individuals is 60%, then the additional 20% (or ? of the total) can be attributed to malaria, and the attributable fraction is 33%. This can also be conceived as the proportion of morbidity that would be removed if malaria were eliminated [24] and can be expressed mathematically as:
Attributable fraction = \( \frac{p_f - p_a}{1 - p_a} \)

Where \( p_f \) = parasite prevalence in febrile subjects and \( p_a \) = prevalence in asymptomatic individuals.

As the proportion of parasitemia in asymptomatic individuals increases, this method loses utility and begins to underestimate the true attributable fraction [25-27]. Also, the precision of the estimate becomes reduced because the confidence intervals widen [26]. Indeed, parasite prevalence in asymptomatic individuals may even exceed that in febrile individuals if fever from other causes suppresses malaria parasitemia [28], giving a negative attributable fraction estimate. Additionally, this method can only provide an estimate of the burden of malarial disease and cannot be used to estimate the proportionate risk that a fever episode in a particular individual can be attributed to malaria.

A more complex mathematical approach based on continuous logistic regression has been developed using quantitative rather than qualitative parasitological data to calculate the overall malaria attributable fraction as well as the likelihood that a particular episode of illness can be attributed to malaria. This method is founded on the assumption that, “the risk of symptomatic malaria increases with parasite density” [24] but, importantly, does not assume that this relationship is a linear one. Illnesses attributed to malaria under this model are those in which the level of parasitemia is raised above that found in afebrile controls. These estimates gain precision over the classical epidemiological approach by using data throughout the range of parasite densities and are therefore less influenced by a small number of aparasitemic individuals. Another advantage is that factors other than parasitemia can be included in the model (such as age and season) and that the sensitivity and specificity of diagnostic criteria such as parasitemia cut-off levels and alternative case definitions can be calculated. It is important to note that the estimates derived from one population apply only to that population and will heavily depend on whether the data was collected by active (e.g., regular monitoring of all individuals in a village) or passive (e.g., health centre presentation of clinical episodes, which may be more severe) case detection. An example of this approach, as it applied to data collected from children aged < 6 years of age from household visits in two
villages in the Kilombero district of Tanzania (where > 90% of children aged > 1 year were parasitemic) is shown in Figure 2.2 through Figure 2.4.

**Figure 2.2. Cumulative distribution of parasite densities with asymptomatic and symptomatic parasitemia**

The cumulative parasite prevalence in febrile (axillary temperature = 37.5°C; unbroken line) and afebrile (dashed line) Tanzanian children as a function of parasite density. The curve in febrile children is lower because very high parasite densities were much more frequent in this group. A higher proportion of febrile children were aparasitemic, which may reflect a suppressive effect of non-malarial fever on parasitemia. Figure reproduced with the kind permission of Tom Smith.
Figure 2.3. Fever prevalence and malaria-attributable fraction

Stippled areas correspond to malaria attributable fevers in the community estimated by the logistic regression model. The hatched area corresponds to fevers not attributed to malaria. The distribution of lower parasitemias (e.g., < 1000 parasites/µL) is similar in both groups, but as parasitemia increases, the likelihood that a fever can be attributed to malaria (i.e., the positive predictive value for that parasitemia as a cut-off) also increases. Figure reproduced with the kind permission of Tom Smith.
Figure 2.4. Sensitivities, specificities and positive predictive values of malaria case definitions based on parasitemia cut-offs

Purpose-specific cut-offs can be determined from this model depending on the sensitivity, specificity and positive predictive value desired. Figure reproduced with the kind permission of Tom Smith.

Numerous other attempts have been made to refine the case definitions used to diagnose uncomplicated malaria in endemic areas with limited success [27]. As seen from the above example, the diagnostic utility of parasitemia in clinical definitions varies inversely with the degree of overlap in parasitemia levels between malaria cases and asymptomatic controls. Fever itself has been examined as a diagnostic marker in the same Tanzanian population from Kilombero [24], where the malaria attributable fraction was defined on the basis of parasitological data collected from individuals detected in household surveys who were divided into those considering themselves sick (based on the opinion of a parent in children < 5 years) or healthy. Using this case definition, the proportion of children with malaria attributable morbidity and an axillary temperature of = 37.5°C was age-dependent, ranging from approximately 33% in infants < 1 year to 76% in children 1-4 years and 37% in those 5-9 years. The authors speculated that a number of factors may have effected their results, including error in measurement of temperature; natural variation in body temperature throughout the day; the effect of time of day and hyperthermia on
axillary temperature in tropical environments; reduced duration of fever during malarial episodes in infancy (possibly reflecting transferred maternal immunity); and a reduction in severity of malaria with advancing age paralleling the acquisition of immunity. The results of studies evaluating various other clinical features have typically not added much certainty to the case definition of malaria in endemic areas [29-35].

2.3.2. Asymptomatic parasitemia

The diagnosis of asymptomatic malarial parasitemia is also potentially problematic as the levels of parasitemia present are frequently much lower than those in clinical malaria and may be so low as to be undetectable by microscopy, which has a sensitivity of approximately 10-20 parasites/µL [36]. Sensitivity for malaria diagnosis appears to be more critically dependent on the experience of the microscopist than specificity, with time spent examining blood smears being another important determinant of accurate classification of smear results [37,38]. Periodic fluctuations in parasite density may increase the likelihood of a microscopically positive smear on one day that was negative on a previous day [22,39], as may rapid fluctuations in density in as little as 6 hours [20]. The prevalence of parasitemia may be heavily underestimated in highly endemic areas, especially in adults in whom anti-parasitic immunity is thought to suppress parasitemias to very low levels. It has even been suggested that in some high transmission regions, the prevalence of parasitemia (as measured by PCR) may be close to universal [21], although this finding will require confirmation in other studies.
Chapter 3. Pathogenesis of malaria

In the main, the pathological consequences of malaria infection derive from the following inter-related events initiated by malaria parasites: loss of erythrocytes; an inflammatory response by the host; and for \( P. falciparum \) only, cytoadherence of parasitised erythrocytes to vascular endothelium. How these events are proposed to interact and cause the recognised disease manifestations and complications of malaria will be outlined briefly in this section, which will be followed by an expanded discussion of toxin-induced pathology.

The characteristic paroxysmal fevers of malaria were ascribed to a malaria toxin as early as 1886 by the famous Italian pathologist and Nobel laureate Camillo Golgi [40]; supposed to result from production of a non-specific physiologically active serum factor in 1969 [41]; proposed to be due to inflammatory mediators such as tumor necrosis factor (TNF)-\( \alpha \) in 1981 [42]; temporally linked to schizont rupture and TNF-\( \alpha \) release in 1989 [43]; shown to be related to increased TNF-\( \alpha \) in the serum of patients with \( P. falciparum \) malaria in proportion to the severity of their illness in that same year [44]; and a similar association between milder disease but higher levels of TNF-\( \alpha \) demonstrated in patients with \( P. vivax \) malaria in 1992 [45].

The degree of anemia in malaria is thought to exceed that which can be explained by destruction of parasitised erythrocytes, leading to the proposition that other factors produced in response to infection may depress erythropoiesis (reviewed in [46]). The pathogenesis of coma in cerebral malaria is also incompletely understood but is likely to involve tissue hypoxia secondary to microvascular obstruction as well as other biochemical processes that alter brain function [47]. These events are thought to be critically dependent on cytoadherence of parasitised erythrocytes to vascular endothelium and localised production of cytokines and other soluble factors.

Together, this initiates a potentially vicious cycle as cytokines such as TNF-\( \alpha \) and lymphotoxin (LT)-\( \alpha \) may up-regulate expression of parasite receptors on endothelial cells [48], thus trapping more parasitised cells and further increasing local cytokine production. It is likely that similar processes lead to failure of other organs [49]. Severe metabolic acidosis with hyperlactatemia is associated with a very high
mortality in malaria [50] and probably results from a combination of tissue hypoxia, direct effects of cytokines, lactate production by parasites and decreased clearance of lactate by the liver [11]. The pathogenesis of other metabolic derangements such as hypoglycemia may share similar antecedents [51].

3.1. Toxic basis of malaria disease

The last decades of the 20th century witnessed a reawakening of interest and a rapid expansion of knowledge in the field of malaria toxicology. This was precipitated by the recognition that malaria and bacterial sepsis share many clinical features and a similar cytokine profile [42], which led to similar lines of scientific enquiry being pursued for both diseases. In 1988 it was demonstrated that injection of purified bacterial lipopolysaccharide (LPS) reproduced the typical syndrome of bacterial sepsis coincident with a rise in serum levels of the pyrogenic cytokine TNF-a [52]. As with bacterial sepsis, it could be suggested that culmination of research into malaria toxicology will rest with the demonstration that a putative anti-toxin therapy has clinical efficacy [53]. How, and how far, research has progressed toward this goal will be outlined in the following chapter.

3.1.1. The role of soluble mediators in malarial pathogenesis

The cytokine response to bloodstream infection with malaria is extraordinarily complex and interpretation of the associated literature extremely challenging. As an example, the list of soluble mediators influenced by malaria infection (as reported in the Medline literature) currently includes: interleukins 1, 2, 3, 4, 5, 6, 8, 10, 12, 13 and 18; interferons alpha and gamma; tumor necrosis factors alpha and beta; lymphotoxin alpha; transforming growth factor beta; prostaglandins D2, E2, F2 alpha and I2; thromboxane and nitric oxide. The available data have been generated from a mixture of in vitro and in vivo studies involving animals and humans as well as their particular cells and parasites. Methods of measurement have varied considerably between studies and individual experiments have necessarily focussed attention on a limited number of responses despite the enormous potential for cross-talk between mediators. Many of these factors have been associated with both pathological and protective responses and there have been numerous instances of conflicting or
unconfirmed results. In all probability, many negative results remain unpublished. Relating this data to the human condition is therefore far from straightforward. Nevertheless, a number of observations first made in the laboratory have been confirmed to have pathological relevance in humans with varying levels of certainty. It is noteworthy though that at the time of writing, there have been no reports of successful therapeutic interventions in humans that have resulted from specific targeting of any one of these soluble mediators.

Rather than review in depth the mechanistic detail associated with regulation of these various responses, the following section will concentrate on the putative pathological role of the cytokine for which there is most evidence of induction by a malaria toxin, TNF-a. The proposed role of NO in malaria pathogenesis will then be outlined after first summarising its biology and immunological functions.

3.1.1.1. Tumor necrosis factor alpha

The importance of TNF-a to malaria pathogenesis was established by genetic association studies after earlier reports had demonstrated a positive relationship between increasing serum levels of TNF-a and severity of malaria [44]. A G?A substitution in the TNF-a promoter region 308 nucleotides upstream of the transcription start site (TNF2) was discovered to be over-represented in Gambian children who died or had severe neurological sequelae from cerebral malaria in 1994 [54]. TNF2 has since been associated with severe disease [55] and increased mortality [56] from malaria in different geographical settings, in addition to mortality from meningococcal disease [57] and septic shock [58]. Importantly, this polymorphism has been functionally associated with increased TNF-a transcription in a reporter gene analysis [59] and other significant TNF-a promoter polymorphisms have also been described that effect outcome from malaria [60]. Despite this reasonably solid evidence, administration of a monoclonal anti-TNF-a antibody to Gambian children with cerebral malaria reduced fever [61] but had no effect on mortality in a large randomised placebo-controlled trial [62]. Administration of polyclonal anti-TNF-a antibodies to Thai adults with severe malaria proved similarly disappointing [63], although the non-significant reductions
in time to clinical resolution apparent in this study may encourage further research in this area.

Reconciling the lack of clinical effect of a theoretically promising therapeutic intervention with the large body of evidence demonstrating numerous pathologically important roles of TNF-α in malaria is difficult but instructive. Prior to the clinical studies, intra-erythrocytic *P. falciparum* had been shown to bind to and up-regulate expression of intercellular adhesion molecule (ICAM)-1 on human endothelial cells *in vitro* [64,65] and co-localisation of parasitised erythrocytes to ICAM-1 had been demonstrated in human brain tissue post-mortem [66]. TNF-α has since been shown to substantially up-regulate ICAM-1 expression on human umbilical vein endothelial cells (HUVECs) *in vitro* [67], although interestingly in the light of recent data (below), the earlier study had shown expression of ICAM-1 to occur independent of TNF-α [65]. It has been suggested that the anti-TNF-α monoclonal antibody used in the clinical studies may have led to retention rather than neutralisation of biologically active TNF-α in the circulation or that once initiated, the TNF-α-induced pathology was irreversible [62]. An alternative explanation may be that TNF-α is not the principle mediator of cerebral pathology. This is supported by the recent demonstration that LT-α derived mostly from intra-cerebral non-leucocyte cellular origins, rather than TNF-α, appears to be the principle mediator of murine cerebral malaria in the *P. berghei* (ANKA) model [68]. This study also demonstrated that TNF-α and LT-α are both required for optimal ICAM-1 up-regulation but that these events did not necessarily predict the development of cerebral malaria. Although animal models differ from human malaria [69], if confirmed in human studies, these findings represent a minor paradigm shift that did not appear foreseeable only one year earlier [47].

### 3.1.1.2. Nitric oxide

The diatomic molecule NO has been suggested to have pathological and protective roles in human malaria and there is evidence to suggest that both may be operative concurrently in certain circumstances (such as cerebral malaria). The biophysiology of NO and its proposed pathological roles are reviewed below, with a detailed discussion of its potential role in immunity to malaria presented in Chapter 5.2.2.
3.1.1.2.1. Biophysiology

NO is the second smallest of all known biological messengers [70]. Its existence in mammalian systems was first hinted at in 1916 [71], then again in 1981 with the demonstration that humans endogenously synthesised nitrates [72] and that rats did likewise independent of the presence of intestinal microflora [73]. Soon after, research into nitrosamine-induced carcinogenesis [74], cardiovascular physiology [75] and innate immunity [76] converged to demonstrate that NO formation was the precursor to endogenous nitrate production and that NO was an essential signalling molecule in human physiology. This work was rewarded by NO being named Science magazine’s “molecule of the year” in 1992 [77] and by its discoverers being awarded the Nobel Prize for Physiology or Medicine in 1998 [78]. NO is synthesised by the reaction of molecular oxygen with a terminal guanidino nitrogen atom of L-arginine, which occurs during the conversion of L-arginine to L-citrulline by one of three isoforms of NO synthase (NOS) [79]. Classification of the NOS isoforms was initially based on a combination of tissue distribution and mode of enzyme regulation. This resulted in the definition of two “constitutive” calcium-dependent isoforms characterised by low-level physiological production of NO (endothelial or eNOS, and neuronal or nNOS) and one “inducible” calcium-independent isoform capable of sustained production of high levels of NO (iNOS). The subsequent discovery of NOS expression by a wider variety of cell types than initially thought, and recognition that under differing conditions each isoform could behave in a constitutive or inducible manner, led to the more meaningful classification of nNOS as NOS1, iNOS as NOS2, and eNOS as NOS3 [80].

NO has been shown to play a variety of roles in human infections, in which most interest has centred on the highly inducible isoform NOS2 and most evidence has accumulated for protective rather than pathological roles [81,82]. NO is a free radical and may act to modulate disease responses either directly or via interaction with a vast array of other molecules due to its readily diffusible gaseous form and propensity to share its one unpaired electron with other molecules [79]. Its numerous effector pathways include regulation of other soluble mediators involved in immune responses (such as cytokines), killing of pathogens, inhibition or promotion of...
apoptosis, regulation of immune cellular differentiation, tissue damage or protection and vasodilation [82-86]. NOS2 is strongly induced by bacterial and parasitic products including bacterial endotoxins and parasite glycosylphosphatidylinositol (GPIs), and strongly inhibited by glucocorticoids [82,87]. Although regulation of NOS2 is complex [88], in general NO production is up-regulated by Th1-type cytokines (such as TNF-α, interleukin (IL)-1, IL-6, IL-12, IL-18, interferon (IFN)-γ and IFN-α) and down-regulated by typically Th-2 responses (including IL-10, transforming growth factor [TGF]-β, IL-4 and IL-13). IgE has also been reported to stimulate peripheral blood mononuclear cell (PBMC) NOS2 expression via interaction with the CD23 receptor of human monocytes [89,90]. Considerable cross-talk in regulation of NOS2 by arachidonic acid metabolites such as prostaglandins (PGs) and of cyclo-oxygenase 2 by NO has been described, with evidence for up-regulatory and down-regulatory interactions in both directions [91,92]. At the molecular level these processes are controlled by signalling molecules and transcription factors including: the Jak-STAT pathway; interferon-regulatory factor-1; mitogen-activated protein kinases; protein kinase C (PKC); phosphatidylinositol (PI)-3 kinase; protein tyrosine phosphatase; protein phosphatases 1 and 2A; IκB /NF-κB and activator protein-1 [93]. Mononuclear cells are a major source of NOS2-mediated NO production in rodents but there has been considerable difficulty in demonstrating high level NO production by human PBMCs [94]. Nevertheless, under appropriate laboratory or field conditions, induction of NOS2 in human PBMCs has been shown to occur [95-98].

As the lifespan of NO as an intact molecule is extremely brief [79], its measurement in complex biological systems raises obstacles. A number of strategies have been adopted to measure NO synthesis at the level of NOS2 gene (NOS2) transcription (messenger RNA [mRNA]), NOS2 protein expression, NOS enzyme activity [L-arginine to L-citrulline conversion assay] and stable breakdown products in urine and serum (nitrite+nitrate [NOx]). The latter is potentially most reflective of actual NO production, but measurement of NOx in humans is confounded by the significant contribution of dietary nitrates to the overall pool [99] and the potential effect of abnormal kidney function on reduced NOx excretion [100]. Failure to account for these two factors makes interpretation of some clinical studies either problematic or impossible. The contribution of homeostatic NO production to overall systemic
levels of NOx is thought to be low [101] in contrast to the high output NO production that typically accompanies NOS2 induction. Studies in US controls have shown that NOS2 induction in human PBMCs under conditions of good health is minimal [95].

3.1.1.2.2. Proposed pathological roles

The evidence to support a pivotal role of NO in the pathophysiology of human cerebral malaria was extensively reviewed and developed into an elegant hypothesis by Ian Clark and colleagues [51]. The crux of this proposal was that sequestered parasites’ toxins activated NOS2 in cerebral endothelial and neuronal cells leading to high output localised NO production, which freely diffused across the blood brain barrier resulting in a reversible comatose state analogous to that of other NO-mediated “comas” such as alcohol excess and general anaesthesia. This idea was supported by early studies correlating higher plasma levels of NOx with increasing severity of cerebral malaria [102-105] and would have been true of Anstey and colleagues’ raw data had it not been corrected for renal dysfunction [96]. The finding that PBMC NOS2 protein was undetectable in cerebral malaria cases in the face of excessive systemic pro-inflammatory cytokine production argued against this hypothesis but did not discount it [96]. Indeed, the general finding that systemic NO production and PBMC NOS expression/activity is inversely correlated with disease severity has been confirmed in other studies [97,106]. At the same time, post-mortem studies of cerebral malaria cases have shown intense staining of NOS2 in cerebral endothelial, neuronal and mononuclear cells [107-109] and one study but not others has shown increased cerebrospinal fluid (CSF) NOx in cerebral malaria fatalities compared to survivors [110].

The findings that NO production is differentially regulated in the brain and in the blood in a murine malaria model [111] and that NOS2 induction is compartmentalised in other infectious disease states [112,113] may provide clues to resolving this apparent paradox. Very recent data further complicates this interpretation. Demonstration in cerebral malaria of profoundly low extracellular concentrations of L-arginine, the essential substrate for NO synthesis by NOS (with levels below the Km for cellular uptake of arginine) likely limits NO synthesis by
tissue NOS in severe malaria [114]. Moreover, at low L-arginine concentrations, NOS enzymatically reduces oxygen to superoxide without producing NO, which has been hypothesised to contribute to oxidative tissue pathology found in severe malaria [114].

It has also been suggested that NO may contribute to malarial anemia given that the pathophysiology of anemia in this setting cannot be attributed solely to destruction of parasitised erythrocytes [46] and that cytokine-induced NO may decrease human erythropoiesis [115,116]. An inverse correlation was shown between urinary NOx excretion and haemoglobin concentration in asymptomatic malaria-exposed Tanzanian children in a regression model controlling for age only, however, the effect of urine NOx disappeared when the significant effect of parasitemia was included in the model [117]. Data from a clinical study purporting to show that increased NO production in children with severe malarial anemia was higher than in children with cerebral or uncomplicated malaria was even less interpretable, as the effect of renal function on NOx levels was not considered [118].

3.1.2. Putative malaria toxins

The basic notion that parasite products released at the time of schizont rupture initiate the characteristic fevers of malaria inspired exploration of whether *P. falciparum* schizonts could stimulate TNF-a production by macrophages *in vitro* [43]. Results from many early experiments were substantially clouded by the realisation in 1998 that frequent *Mycoplasma* contamination of *P. falciparum* in continuous culture may have been wholly or partly responsible for cytokine induction [119,120], as *Mycoplasma* lipoproteins are potent inducers of TNF-a [121]. Nevertheless, it was later proven that human monocytes produced large amounts of TNF-a *in vitro* following rupture of co-incubated *P. falciparum* schizonts from *Mycoplasma*-free culture [122]. Despite the early problems with *Mycoplasma*, GPI molecules were identified during this period as principle candidate toxins through various physical and chemical extraction procedures [123] and by reproduction of a compatible malaria syndrome following administration of semi-purified GPI to mice [124].
Although interest in GPIs dominates the field, others have suggested that hemozoin (also known as “malaria pigment”; an aggregation of insoluble heme groups released from the digestion by trophozoites of host hemoglobin as well as the remnants of host and parasite membranes [125]) fulfils a role as a cytokine-inducing malaria toxin. A number of the studies suggesting toxicity were conducted in the period preceding identification of *Mycoplasma* contamination of parasite cultures and used varying purification procedures [126-129]. Other studies produced contradictory results [130], although it has been suggested this may be due to oxidative stress depressing phagocyte function that is induced by ingestion of pigment in some instances [131]. A recent paper described TNF-a production by human PBMCs in response to de-proteinated hemozoin enriched from *P. falciparum* culture, although it is unclear whether all other membrane contaminants were removed from this preparation or if the cultures were monitored for *Mycoplasma* contamination [132]. A similar paper had shown considerable loss of activity following de-proteination of hemozoin several years earlier [126]. Somewhat surprisingly, research in this area does not appear to have advanced far in the last 5 years, despite the availability of chemically synthesised ß-hematin, a structurally identical analogue of the core component of malarial hemozoin [133].

### 3.1.2.1. Glycosylphosphatidylinositol molecules

Glycosylphosphatidylinositol molecules are present in the outer membranes of a wide variety of eukaryotic cells, including those of yeasts, protozoa and mammals. The core structure of GPIs has been found to be remarkably conserved among all species studied thus far (Figure 3.1), comprising a single membrane associated phospholipid head, to which is attached (in order): a phosphodiester-linked inositol ring; a glucosamine (GlcN); a linear chain of 3 mannose sugars (Man); and a phosphoethanolamine (P-EtN) linked to the terminal Man residue [134]. The most fundamental function of GPIs is “to afford the stable association of proteins with the surface membrane lipid bilayer” [135] via an amide bond linking the carboxy-terminal residue of the protein to the amino group of P-EtN. Over 100 different protein associations were described between 1985 and August 2002, of which the only common feature is anchorage to the exoplasmic leaflet of the membrane by GPI [134]. At least 15 different proteins appear to be GPI-anchored in *P. falciparum,*
including merozoite surface protein (MSP)-1, MSP-2 and MSP-4 [134,136]. GPIs may also be found free of protein attachment in the outer membrane in *P. falciparum* [137] and in other parasites [135]. Variation between the GPIs of different species relates to the presence, number and type of accessory carbohydrate and EtN branches attached to the mannose residues; the optional attachment of an acyl (fatty acid) chain to the *myo*-inositol ring; the type of lipid backbone (glycerol versus ceramide) and bonds used to attach hydrocarbon chains (ester versus ether); and the number, length and degree of saturation of the hydrocarbon chains [135].

### 3.1.2.1.1. Structure and biosynthesis of *P. falciparum* GPIs

Unlike higher eukaryotes such as mammals, post-translational glycosylation of proteins in *P. falciparum* occurs predominantly, if not exclusively, by attachment to GPI anchors [138,139]. The complete structure of *P. falciparum* GPIs has been elucidated by direct biochemical analysis and mass spectroscopy and shown to differ from human GPIs and those of other parasites in a number of respects (Figure 3.1; [140]). In contrast to human GPIs, *P. falciparum* GPIs appear to always have an acyl chain attached to the inositol ring, which may vary in composition to that of mammals [137,141] and is often lacking from other protozoa [142]. Human GPIs characteristically have at least one additional EtN moiety as well as other carbohydrate moieties attached to their glycan core (completely lacking in *P. falciparum*) and differ with respect to the type of fatty acids present in the diacylglycerol moiety. *P. falciparum* characteristically has an additional 4th mannose residue attached to the terminal mannose of the glycan core that is variably present (but usually absent) in human GPIs [142]. Importantly, the structure of *P. falciparum* GPIs appears to be conserved across a wide variety of different geographical isolates [143].
Figure 3.1. The proposed structure of *P. falciparum* GPIs

The core structure consists of the membrane associated phospholipid head (bottom right; a diacylglycerol moiety in *P. falciparum*) attached to a phosphodiester-linked inositol ring, glucosamine (GlcN), a linear chain of 3 mannose sugars (Man) and a phosphoethanolamine (P-EtN) linked to the terminal (left) Man residue. Proteins are commonly linked via an amide bond to the carboxy-terminal residue of the protein to the amino group of P-EtN (top left). The fatty acids and their molar proportions are indicated. In *P. falciparum*, the inositol associated acyl chain is most commonly palmitate with minor proportions of myristate (which prior to the year 2000 had not been identified on the inositol ring of other GPIs). The diacylglycerol comprises unsaturated acyl substituents at *sn*-2 (major C18:1 and minor C18:2; left) and predominantly C18:0 and a range of variable size saturated acyl residues at *sn*-1 (right). The presence of a 4\textsuperscript{th} Man (bottom left) and a glycan core free of extra P-EtN and carbohydrate residues is uncommon in human GPIs. Figure and legend reproduced with kind permission of D Channe Gowda [140].

A detailed understanding of the processes involved in biosynthesis of GPIs by *P. falciparum* is a pre-requisite if this metabolic pathway is to be therapeutically targeted. The dissection of these complex processes has progressed rapidly in recent years, leading to the description of 8 new *P. falciparum* genes predicted to encode essential GPI-synthetic enzymes [144]. Synthesis of *P. falciparum* GPIs has been shown to occur exclusively during the trophozoite stage by treating synchronous cultures with selectively timed administration of mannosamine, an inhibitor of GPI
biosynthesis [145]. Furthermore, mannosamine was capable of completely arresting trophozoite development in the same growth cycle, as well as revealing a sequence of GPI assembly that was uniquely different to that described in other parasites and humans. Collectively these observations have facilitated the identification of several potential \textit{P. falciparum}-specific steps in the biosynthetic pathway that might enable selective targeting by novel pharmacological agents.

3.1.2.1.2. Function and mechanisms of action of \textit{P. falciparum} GPIs

Interest in \textit{P. falciparum} GPIs has centred mainly on their potential to induce cell-mediated pathological and immune responses, however, GPIs may also alter the conformation of attached proteins in a manner that influences protein function and immunogenicity [146]. This may have important implications for the development of vaccine epitopes based on recombinant protein antigens, some of which to date have been based on GPI-anchored proteins [136].

It has recently been demonstrated that murine antibodies raised to a non-toxic chemically synthesised \textit{P. falciparum} GPI analogue with no acyl chains completely abolished whole schizont extract-induced TNF-\(\alpha\) release from mouse macrophages [147]. To the extent that the \textit{in vitro} model matches the clinical situation in humans, this study provides the best evidence to date that GPIs are the predominant pro-inflammatory toxins of \textit{P. falciparum}. This finding also consolidates earlier evidence showing that both monoclonal and polyclonal murine antibodies raised to purified parasite GPI had neutralising activity [148]. In addition to invoking production of TNF-\(\alpha\) by mononuclear cells, \textit{P. falciparum} GPIs have also been shown capable of inducing macrophage production of IL-1 [124], as well as production of NO by macrophages and vascular endothelial cells in a process enhanced by IFN-\(\gamma\) [149]. In similar experiments, \textit{P. falciparum} GPIs up-regulated expression of ICAM-1, vacular cell adhesion molecule-1 and E-selectin in HUVECs both directly, and indirectly secondary to TNF-\(\alpha\) and IL-1 production [150]. This process was also blocked by monoclonal anti-GPI antibodies. \textit{P. falciparum} GPIs have also been shown to possess insulin-mimetic activity, causing increased glucose oxidation in murine adipocytes \textit{in vitro} as well as inducing TNF-\(\alpha\)-independent hypoglycemia following intraperitoneal administration to thioglycollate-primed mice.
Taken together, the data from this elegant series of studies directly link *P. falciparum* GPIs to a number of critical synergising events potentially involved in human malarial pathogenesis.

Induction of TNF-α production in macrophages by *P. falciparum* GPIs requires the sequential activation of two distinct but coupled cellular signals: the first initiated by the distal 4th mannose residue and the second by the sn1-acylglycerol moiety [151]. Removal of either component completely abrogates TNF-α production and deacylated carbohydrate moieties can effectively inhibit the toxic activity of GPIs. Although the cellular receptors and mode of interaction with GPI have not been precisely determined, the process is thought to be novel in that it does not appear to involve insertion of GPIs into the plasma membrane nor endocytosis [151]. It is also unclear to what extent GPI induction of TNF-α production in humans may occur through direct stimulation of mononuclear cells versus a requirement for CD3+ T cell co-operation [122]. More is known of the subsequently triggered intracellular events, which involve initial activation of protein tyrosine K (PTK) by the terminal mannose followed by acylglycerol-induced activation of PKC [149-152]. These pathways collaboratively regulate production of the nuclear factor (NF)-?B, which is a key inducer of transcriptional activation for a number of pro-inflammatory cytokines [153]. Inhibitors of either PTK or PKC can block GPI-induced activation of TNF-α, NO and endothelial cell receptor up-regulation [149-152]. The apparent uniqueness of the cellular activation process, together with the demonstration that several steps can be inhibited, suggests another avenue through which *P. falciparum* GPIs may be therapeutically targeted.

3.1.2.2. *Plasmodium vivax* toxins

The predilection of *P. vivax* for selective invasion of immature erythrocytes is probably the major reason for the lack of success in establishing an *in vitro* continuous culture of *P. vivax* beyond 5 or 6 asexual cycles [154]. Thus, little progress has been made in characterising the toxins of *P. vivax*. Nevertheless, a number of parallels suggest that the toxins of *P. vivax* and *P. falciparum* are at least functionally similar. Mild to moderate illness caused by the two parasites may be so similar as to be clinically indistinguishable in areas of roughly equivalent endemicity.
The dynamics of fever in relation to production of schizont rupture and TNF-a production follows the same pattern for both parasites and TNF-a production may even be higher for *P. vivax* than for *P. falciparum* [45]. Serological cross-reactivity against the TNF-a-inducing activity of *P. falciparum* and *P. vivax* was apparently demonstrated *in vitro* and the TNF-a blocking activity of serum derived from a patient with clinical *P. vivax* infections was ablated by pre-treatment with PI liposomes [156,157]. More recently, antibodies raised to a fully synthetic *P. falciparum* GPI subcomponent inhibited toxicity induced by *P. berghei* in rodents [147]. This suggests that significant functional and structural similarities may exist between the toxins of different *Plasmodium* species. The recent identification of GPI-encoding homologue genes of *P. falciparum* [144] and the ongoing sequencing of the *P. vivax* genome [158] may soon provide another avenue for comparison.

### 3.2. Strain differences in parasite virulence

Classical teaching dictates that the likelihood of developing illness from an infectious disease is directly proportional to the inoculum and virulence of the organism and inversely proportional to the host’s resistance. In one of the earliest reports describing the introduction of *P. falciparum* for malariotherapy of neurosyphilis it was stated that, “a striking difference can… be observed between the clinical virulence of different geographical races or strains… and… the difference is most apparent between various races of *P. falciparum* [cf *P. vivax*]” [159]. Different “strains” were generally defined on a geographical basis at that time, which was recognised as a “loose” classification [160] but nevertheless was suitable for defining isolates that varied according to their “biological characteristics and in the severity of the ensuing disease” [12,161]. The subsequent identification of parasite virulence determinants has been hampered by a lack of descriptive epidemiological studies of clinical disease, difficulty in defining parasite population structures in endemic areas and a general inability to develop strain-typing systems that better correlate with virulence than the geographical classification [12]. Mathematical modelling has suggested that few “strains” cause cerebral malaria, although this technique cannot identify the physiological basis underlying strain classification [162]. The advent of molecular technology though has led to the ability to identify “parasite polymorphisms affecting critical parasite functions and those governing the
parasites’ ability to avoid or suppress the host immune response” [12]. Hence, variant expression of *P. falciparum*-determined erythrocyte surface antigens involved in cytoadherence [163-165], and the ability of some strains but not others to induce non-infected erythrocyte clumping known as rosetting [166] or to inhibit maturation of dendritic cells involved in the earliest stages of the immune response [167], have been proposed as likely virulence determinants. The “intrinsic parasite multiplication rate”, a contributor to the rate of expansion of the parasite biomass [168], is another potential virulence factor that has recently been identified as correlating with the severity of human malaria [169]. A “strain’s” ability to induce cytokines is another logical virulence determinant and apparent geographical strain-related differences in the parasite density at first fever in non-immune neurosyphilis patients treated with malariotherapy supports this view [170]. Further corroborative evidence was provided by experiments that examined human mononuclear cell TNF-a production *in vitro* in response to different *P. falciparum* strains [171,172], however, the report from the same team of widespread contamination of *P. falciparum* cultures by *Mycoplasma* casts considerable doubt on this finding [119].
Chapter 4. Immunity to malaria

In 2002, it is probably just as true to say as it was 100 years ago that the best correlate of natural immunity to malaria is the presence of malaria parasites in the blood of individuals without clinical symptoms of malaria. This remains so despite extraordinary advances in the understanding of malariology and immunology (including over 2850 Medline publications alone combining MeSH headings of “malaria” and “immunity”). It can be concluded from these two statements that natural immunity is imperfect and that the mechanisms governing it are likely to be multifactorial and involve complex interactions. One approach to untangling these inter-related processes is to first ask what they do (below), and then ask how they do it (Chapter 5).

It is generally accepted that malaria-naïve subjects possess little effective immunity and that the degree of protective immunity in malaria-exposed subjects correlates with the intensity of exposure to transmission. It is also apparent that cumulative exposure may result in an increase in protective immunity over time, although separating the possible effect of exposure from that of age per se is not straightforward. A special case exists in newborn infants, who for the first 6 months of life enjoy a period of protection at a level roughly equivalent to that of the mother. Immunity to malaria may be lost at any age if exposure is interrupted, in as little as a matter of months. It appears that complete sterilising immunity to malaria (the ability to prevent the establishment of new infections or to rapidly and completely eliminate new infections) is never achieved. The obvious conclusion to be drawn is that protective immunity against malaria requires ongoing exposure to be maintained, and is relatively short-lived compared to that of many other infectious diseases.

That asymptomatic infections can become established, and persist at reasonably stable densities over time [39,173-175], implies the existence of immune processes that limit exponential expansion of parasite numbers without eradicating parasites completely. As this stable density is usually higher in children than in adults from endemic areas, it may be inferred that the mechanisms limiting exponential expansion are for most of the time operating at least as actively in children as in
adults, regardless of their potential overall capacity. It appears that this presence of parasitemia is an effective barrier to the establishment of superinfections for much of the time, a phenomenon accurately termed premunition. Nevertheless, this immunity can be overcome from time to time under the right conditions, more easily in children than in adults from highly endemic regions, resulting in symptomatic clinical disease. Furthermore, in the event of symptoms, young children appear least able to limit the severity of disease compared to older children and adults, both in respect to toxic manifestations and those primarily related to cytoadherence of parasites to endothelium.

A reasonable conclusion that can be drawn from the persistence of asymptomatic infection is that another form of immunity exists enabling hosts to prevent symptoms from developing following the release of pyrogenic toxins coincident with schizogony. This state has been referred to as “tolerance” of malaria parasitemia (or “malarial tolerance”) and has been fairly said to “go through historical phases of popularity” [176]. The concept had its genesis in the observations of classical malariologists seeking to reconcile the apparent good health of young children especially with levels of parasitemia apparently sufficient to cause illness in other settings [177,178]. The existence of such immune processes requires demonstration that the tolerated parasites are otherwise fit and capable of causing disease and that the levels of parasitemia tolerated would indeed be sufficient to cause illness under different conditions. These questions are addressed in more detail in Chapter 4.2.

Populations living under similar conditions with respect to malaria transmission intensity often vary from one another in relation to the spectrum of malarial disease that is seen, and individuals from within the same population vary from one another in their susceptibility to disease. Theoretically, this may in part be explained by subtle differences in malaria exposure and parasite strains. Additionally, constitutional or “genetic” differences between human hosts are known to exist that modify the immune responses governing the outcomes of the host-parasite relationship. Moreover, exposure to other infections and antigens that vary between populations and individuals may also potentially generate immune-responses capable of modifying the immune response to malaria.
4.1. Terminology

Numerous terms have been used to define various aspects of immunity to malaria (in addition to those mentioned above) including, innate, acquired, specific, non-specific, anti-parasitic, clinical, anti-disease and anti-toxic. Given the overall complexity of anti-malarial immunity, it is not surprising perhaps that confusion exists over the use of some of these terms. As an example, “anti-disease” immunity has been used specifically to describe that which acts by “reducing parasite densities in subsequent infections” [13], whereas the same term has been used more generally to describe immunity “which ameliorates disease despite the persistence of circulating blood-stage parasites” [179]. As all forms of immunity to malaria act to prevent disease, by definition, use of this term (and the related term “clinical” immunity) will be discarded from future use in this thesis. To avoid confusion, the following terms will be used as defined by Kevin Marsh in Bruce-Chwatt’s Essential Malariology [176] with one modification. “Specific” immunity refers to that acquired in response to malaria infection itself, whereas non-specific immunity requires no previous exposure to malaria and may be either “innate” (e.g., genetically determined) or “acquired” (e.g., in response to other infections/antigens). In this thesis, the definition of “specific” will be restricted to responses induced by malaria that primarily recognise malaria, so that malaria antigen-specific immune responses can be distinguished from modification of non-specific mechanisms by malaria. It should be recognised that non-specific immune responses may exert an influence on the acquisition of specific immunity.

The terms “anti-toxic” and “anti-parasitic” immunity encompass various, but not all, aspects of specific and non-specific immunity. For the purpose of this thesis, anti-parasitic immunity includes immune responses that either inhibit parasite replication or growth, or result in parasite death. Premunition, maintenance of stable parasite densities over time, killing and removal of parasites can all be considered as examples of anti-parasitic immunity and will not be considered in detail here. Instead, the reader is referred to a number of excellent reviews on these topics [174,176,180,181]. In this thesis, anti-toxic immunity refers to responses that act by neutralising malaria toxins or inhibiting the subsequent toxin-triggered human (host) responses. In the context of the previous section, limitation of malarial disease severity in the setting of clinical infection, in so far as it relates to disease
manifestations thought to be toxin-initiated, can be considered as a form of anti-toxic immunity. “Tolerance” can be considered as another form of anti-toxic immunity, and will be used in its narrower sense to define the immunity proposed to mediate resistance to symptoms (particularly fever) in the setting of asymptomatic blood-stage parasitemia.

4.2. The nature of anti-toxic immunity to malaria and evidence for its existence

4.2.1. Observational studies of the relationship between parasitemia and fever

4.2.1.1. Historical studies – definition of “tolerance” and the “pyrogenic threshold”

Working in India, Major Sinton and colleagues [182] measured *P. vivax* densities during periods of health and illness in 50 British soldiers with chronic relapsing malaria. After reviewing the available literature, they observed that a pyrogenic threshold of approximately 5000 parasites/µL existed in their population, which far exceeded the “usual limits” of approximately 200-500/µL reported in “fresh infections”. While noting that the peripheral parasitemia during fever was an imprecise estimate of the number of bursting schizonts, they interpreted their data as supporting the view that, “a tolerance to the clinical effects of the infection has been developed in the former infections” [182]. This accorded with the earlier view of Ross and Thompson (1910; quoted in [182]) that, “it is probable also though by no means certain, that the resistance to the toxins of the *Plasmodia* varies, not only in different persons but in the same person at different stages in the course of his infection and under different physiological conditions.” The possibility that genetic determinants may underlie an individual’s ability to resist malaria toxins was recognised by Lieut.-Colonel Sinton in 1938, when he rather bombastically observed that, “it appears to be due to a weeding-out of the more susceptible units by death in early life and so the eventual production of a more resistant race” [177].

Further evidence to support the intuitions of Sinton, Ross and Thompson was garnered by Boyd in 1938 after following the clinical course of 77 untreated induced primary attacks of *P. vivax* [183]. All but 5 subjects had an initial “pyretogenic
density” of = 500 parasites/µL, whereas only 7 subjects had a parasitemia below this density on the day of their final pyrexia (median density 2000-4000 parasites/µL after a median of 29-42 days untreated infection). Boyd interpreted his data to, “clearly indicate that before the body fully acquires the ability to destroy the parasites it has become markedly tolerant to their presence.” Almost identical results were reported from a larger group of patients by Kitchen in 1949 [184]. In 1948, Blackburn (quoted in [185]) refined this supposition by suggesting that such tolerance was strain-independent in induced infections, in contrast to the delayed acquisition of strain-specific antiparasitic immunity. Further recognition that genetic host differences may also be important was given by Kitchen who stated that, “the duration of clinical activity in white patients [given malariotherapy for neurosyphilis] is usually greater than that in colored persons, in whom we recognise a measure of inherent tolerance” [186]. This was reflected in the recommendation that *P. falciparum* rather than *P. vivax* should be given on the primary inoculation to, “[neurosyphtilic] subjects raised near the Mediterranean sea… persons who have spent time in an endemic region… and all colored persons”[187].

In an oft quoted study, Miller [185] followed a highly malaria-exposed cohort of 20 Liberian adults aged 20 to 30 years with second daily blood films for one year, along with 10 children aged 3 to 7 years whom he followed similarly for a period of 71 days. Daily blood films were prepared upon the detection of parasitemia and continued until 3 days had elapsed following infection, at which time second daily smears were resumed. Temperatures were recorded at the time each blood smear was made, clinical histories were maintained throughout his investigation and episodes of fever were not treated. The mean parasite count at the initiation of febrile episodes due to *P. falciparum* was 1644/µL (range 30-4550; geometric mean (GM) and 95% confidence interval (CI) 881 [523-1487]) in adults and 1837/µL (range 11510-37210; GM and 95% CI 17277 [13253-22522]) in children. Children’s average daily parasite count during the low transmission season (2230/µL; GM 956) exceeded the majority of the adults’ pyrogenic thresholds. In conclusion he noted that, “while adults were more efficient in suppressing parasite levels and suffered less from clinical attacks of malaria, children could tolerate higher parasite burdens without showing clinical evidence of disease.” He coined the term “detoxifying
resistance” to explain the ability of children to “detoxify those products of parasite metabolism responsible for the malaria paroxysm.”

4.2.1.2. Regions of high malarial endemicity

Trape and colleagues [34] combined observations made in different villages during different time periods to estimate the pyrogenic thresholds of Congolese children (< 15 years) exposed to intense perennial malaria transmission. Temperatures were only taken from children complaining of fever or other symptoms in the first village, whereas temperatures were systematically recorded periodically in the second. A rectal temperature = 37.5°C was used to define fever in the first population (one third of these febrile subjects had temperatures in the range 37.5-37.9°C), whereas an axillary temperature of = 38°C was used in the second. Notably, axillary temperatures are generally considered to be slightly lower than concurrent rectal temperatures [34,188]. In children from the first population (ages 0-14 years; all of whom were febrile), there was a higher proportion of children thought to have malaria on clinical grounds with parasitemias > 5000/µL (68%) than in those thought to have other diseases (19%). In the second population, 61% of febrile children (aged 4-14 years) had a parasitemia > 5000/µL compared to 20% of afebrile controls. Because febrile children thought not to have malaria from the first population had a similar distribution of parasitemias to the afebrile group from the second population, it was assumed that a diagnosis of malaria could be discounted in those with parasitemias < 5000/µL. Notwithstanding the substantial methodological anomalies present in this study, this figure has been quoted in other studies as representative of a discriminatory pyrogenic threshold in this population [13,30,33,189-192].

With the aim of describing malaria morbidity, Velema and colleagues [189] surveyed 1500 children from Benin aged < 3 years with monthly household visits directed at uncovering children with fever (rectal temperature = 38°C). Blood smears from subjects and age-matched afebrile controls from the same village who were visited on the same day were subsequently prepared in the hours following temperature measurement. There was little variation in morbidity over the 10 months of the study (conducted during the long rains, short dry and short rainy seasons). In 325 cases of fever identified from almost 13000 visits, 58% of subjects were parasitemic
compared to 41% of controls. Of the thresholds considered, a parasite density of 1000/µL was the most discriminatory, with 28% of febrile subjects exceeding this threshold compared to 5% of controls (odds ratio [OR] 7; 95% CI 4-13; P<0.001). This threshold was exceeded by 49% of parasitemic subjects, compared to 13% of controls (OR 6; 95% CI 3-12; P<0.001). Notably, field-workers received a monetary bonus for each case of fever that was identified in this study.

For a period of 3 years, Rooth and Bjorkman [30] ran the only clinic in a coastal Tanzanian village where malaria transmission was perennial and holoendemic and collected clinical and parasitological data on all children aged 1-9 years presenting with fever. In marked contrast to the Congolese study [34], they found significantly higher parasitemias in children with asymptomatic *P. falciparum* infection (recruited during malarial surveys) than in febrile children with clinical diagnoses other than malaria. A clinical diagnosis of malaria was defined on the basis of any parasitemia plus fever (axillary temperature > 37.5°C) in the absence of clinical features suggestive of another illness. In febrile children, a cut-off of 400 parasites/µL had a sensitivity of 96% and specificity of 92% for the diagnosis of malaria. It is noteworthy though that in this study, 79% of the febrile cases presented longer than 24 hours after the onset of symptoms and that multiple observations from the same child were used in the same statistical analyses. Also, although GM parasitemias in malaria cases aged 0-4 years were more than double those of older children, the effect of age on pyrogenic thresholds was not investigated and age was not considered as a possibly confounding variable in any of the statistical analyses.

Using a similar method of passive case detection by nurses at a local clinic [31], Genton and colleagues investigated 2096 presumptive malaria cases in the Wosera region of perennial and high transmission in PNG and collected malarialmetric data during the same time period. Although the primary purpose was to examine the utility of clinical diagnostic criteria, from the data presented 70% of children with presumptive malaria (defined by a study nurse on the basis of fever history without an obvious alternative diagnosis) and *P. falciparum* parasitemia had = 5000 parasites/µL on blood smear compared to 18% of asymptomatic parasitemic control children. In similarly defined adult cases = 15 years, 55% had = 1000 parasites/µL.
compared to 14% of asymptomatic parasitemic controls. This suggests that the sensitivity and specificity of different cut-offs varies according to age in accordance with Miller’s observations from Liberia [185]. In a prelude to a vaccine trial, Beadle and colleagues [191] pharmacologically cleared parasitemia in Kenyan children aged 0.5-6 years and then closely monitored the 92% of their cohort who were aperasitemic 14 days later for a total of 84 days. The incidence of fever (temperature = 37.5ºC per axilla; = 37.8ºC oral/rectal) at the time of first parasite recurrence was similar in children with < 5000 \( P. falciparum \) parasites/µL (10%) to that associated with multiple negative blood smears collected from the same children (6%), suggesting that the proportion of malaria-attributable fevers in infected children was small. In contrast, 26% of children with 5000-19999 parasites/µL were febrile, as were 64% of those with = 20000 parasites/µL. It is unclear whether age-defined cut-offs might have increased the sensitivity and specificity of malaria diagnosis or whether the marked differences in the density of recurrent parasitemias that were observed in relation to the season of enrollment may have affected this interpretation.

A cohort of 241 children aged between 2 and 15 years was surveyed longitudinally for 1 year to determine the relationship between parasite prevalence and density, and malarial disease along the Gogol River Basin in Madang Province on the north coast of PNG [13]. Malaria transmission in this region is intense and both \( P. falciparum \) and \( P. vivax \) are endemic [193]. Temperatures and morbidity surveys were recorded weekly in each child, in addition to malarometric surveys at 3 month intervals and blood smears prepared if illness was or had been present within the previous 12 hours. The prevalence of asymptomatic parasitemia for both species was highest in children aged 5-9 years, whereas both parasite densities and fever (on history and/or axillary temperature > 37ºC) incidence were highest in children aged 2-4 years and decreased thereafter with age. In self-reporting febrile cases, there was a significant negative association between age and parasite density, which suggested that the pyrogenic threshold decreased with age. Transition from an afebrile to a febrile state was associated with an increase in parasite density in 71% of children, and transition from a febrile to an afebrile state was associated with a decrease in parasitemia in 68% of children. Transitions between consecutive afebrile states in contrast were associated with a normal distribution of parasite intensities around zero.
Bouvier and colleagues [35] enrolled approximately 800 children aged 1 to 12 years in a rural village in Mali where 6 months of intense malaria transmission during the wet season is abruptly halted during the dry season. Children were examined and fingerprick blood smears prepared on 10 consecutive days during the end of the dry season and again at the peak of the transmission season. In this ethically questionable study, children with fevers (oral temperature > 38°C) and no obvious signs of severe malaria or non-malarial infection were treated only with paracetamol - anti-malarial treatment was withheld until after follow-up. The 9 day cumulative fever incidence in children afebrile on the day of initial enrollment was 2% in the dry season and 8.2% in the wet season, with the incidence of non-malarial fevers similar in both seasons (1.1% vs 1.3% respectively). During the wet season, the relative risk of fever in children age = 4 years with a parasitemia > 15000/µL was 2.7 (95% CI 1.4-5.4), however, only 18.9% of those with parasitemias above this cut-off were febrile, as were 7.5% of those with lower parasitemias. In children aged < 4 years, any parasitemia was associated with an increased risk of fever. A similar but less impressive trend was seen at a cut-off of 2000 parasites/µL during the dry season for children of all ages.

The definition of pyrogenic thresholds for *P. vivax* in endemic areas has received less research attention than for *P. falciparum* but there is preliminary evidence to suggest that *P. vivax* fever thresholds might decrease with age. Although a single cut-off of 500 parasites/µL was able to define a clinical case of *P. vivax* in a cohort from the Solomons aged 1-9 years with a sensitivity of 93% and a specificity of 95%, it appeared from the data presented that *P. vivax* parasitemias associated with symptoms were lower in those aged 5-9 years compared to 1-4 years [194]. This effect was independent of season but not analysed statistically. In the dry season, the GM parasitemia in fever cases was 2550/µL for children aged 1-4 compared to 1250/µL for those aged 5-9 years. In the wet season, the corresponding levels were 3300/µL and 2450/µL respectively. Interpretation of this data is made more difficult by the possibility of density-regulating inter-species interactions between *P. falciparum* and *P. vivax* [173], which apparently resulted in statistically fewer mixed species infections in the Solomons study than were expected.
4.2.1.3. Regions of low to moderate malarial endemicity

The positive predictive value of parasitemia as an indicator of disease is increased in areas of low endemicity compared to regions of high endemicity and the age range of symptomatic and severe disease is broadened [32]. Working with displaced Karen refugees along the Thai-Burmese border, Luxemberger’s group [32] monitored malarial morbidity in a cohort of children aged 4 to 15 years exposed to *P. falciparum*, *P. vivax* and *P. malariae* with regular parasitological and clinical surveys for 1 year. Most episodes of parasitemia were either associated with symptomatic malaria (defined on the basis of typical clinical features in the absence of an alternative diagnosis) or followed by symptomatic malaria within 1-3 days, which was more common for *P. falciparum* (84%) than for *P. vivax* (57%). The GM parasite densities at the onset of symptoms (usually fever) were 1460/µL (95% CI 327-6516) for *P. falciparum* and 181/µL (95% CI 45-734) for *P. vivax*. The authors interpreted these levels as being roughly equivalent to those reported in naïve subjects [184]. Sowunmi’s group examined malarial morbidity in relation to asymptomatic parasitemia in urban [19] and rural [175] Nigerian children exposed to moderate and high endemicity respectively and found that 47% of urban children with malarial symptoms had a parasitemia < 1000/µL, whereas all rural children with parasitemias of this level were asymptomatic. While this value is not representative of a threshold and the studies were temporally separated, it is suggestive of a relative difference in tolerance between the two groups.

4.2.1.4. Induced infection of malaria-naïve patients and subjects

It was stated in 1949 that, “The accepted use of malaria infections as therapeutic agents in the treatment of neurosyphilis may be fairly said to have been responsible for a renaissance in clinical malariology, for it has contributed immeasurably, if indirectly, to our systematic knowledge concerning the natural evolution of these infections” [184]. In 1999, a bioethicist concluded that the acquisition of knowledge from malariotherapy records was “ethically supportable” on the grounds that malariotherapy was standard practice at the time, and that the only additional research interventions involved extra blood tests and centralised data recording.
Consent had been given by the families of the patients with neurosyphilis or the courts. The conduct of research involving experimentally induced infection of “volunteer” prisoners may have been less ethical if it involved exploitation of participants, notwithstanding the potential value and scientific validity of the work. Examination of such databases has to date resulted in two studies addressing specific questions central to the acquisition of anti-toxic immunity.

Gatton and Cheng [197] analysed previously unpublished data collected between 1931 and 1963 from neurosyphilis patients resident at the South Carolina and Georgia State Hospitals, and between 1962 and 1974 from prisoners of the Illinois State Penitentiary. Their purpose was to identify “the level of [*P. falciparum*] parasitemia required to induce a fever response and the changes in this threshold early in an infection.” In malaria-naïve patients, the pyrogenic threshold associated with the first fever of the first infection varied according to the strain of parasite used, from a GM of 656/μL for the McLendon strain (similar at both sites) to a combined mean of 2594/μL for the El Limon and Santee Cooper strains. The initial pyrogenic threshold parasite density was approximately 2.5-4 times higher in prisoners of African descent than in Caucasians for 2 of 5 strains in whom sufficient comparative data was available (the Malaya and Uganda strains). There was a significant increase in pyrogenic threshold between the first and second fevers for all three strains in the hospital dataset, but no change for the remainder of the initial infection. The pyrogenic threshold associated with the onset of a second infection in reinfected prisoners (n=58) was significantly increased and approximately double that of the first (mean parasite-free interval 186 days). In a smaller subset of neurosyphilis patients (n=23), there was a similar increase in magnitude of the pyrogenic threshold between first and second infections (mean parasite-free interval 152 days), but this was not statistically significant. The increased overall pyrogenic threshold seen in prisoners was also evident in the subset re-challenged with a heterologous strain (n=35) but not in the smaller subset with a homologous strain (n=23). There was no correlation between the changes in pyrogenic thresholds from one infection to the next and either the duration of the primary infection or of the parasite-free interval between infections.
Molineaux and colleagues [198] analysed data from patients with neurosyphilis collected during the same era as that utilised by Gatton and Cheng to compare *P. falciparum* densities on the first day of fever in a primary and secondary induced infection. They found no significant change in the pyrogenic threshold density between primary and secondary infections in 29 of the 38 patients who became febrile during both infections. Seven of the 9 remaining subjects failed to develop a fever during the second induced infection despite a higher parasite density (1.4-6.1 times higher than the primary infection) in 5 subjects. In addition, 6 of the 38 patients became febrile with subpatent parasitemia during the primary infection, compared to none during the second infection. The authors interpreted these last two points to suggest that anti-toxic immunity had developed between the first and second infections, independent of the development of anti-parasitic immunity (which was examined independently).

### 4.2.2. Mathematical modelling of the relationship between parasitemia and fever

Smith et al [25] used logistic regression to model the risk of clinical malaria as a continuous function of parasite density across a broad age range of 0 to > 40 years in the Wosera region of PNG (northwest of Madang). Data were collected from 736 presumptive malaria cases (diagnosed on clinical grounds by a nurse at a health centre on the basis of fever history in the absence of non-malarial features) and 1708 healthy controls recruited in community surveys. Age group terms were included in the model and proved highly significant. The overall malaria attributable fraction (Chapter 2.3.1) in the presumptive cases was 37% and peaked in children aged 2-4 years, after which it declined with age. This suggests that an excess of non-malarial illnesses was likely in infants < 2 years and that anti-parasitic immunity increased with age beyond 4 years in parallel with the demonstrated decreased incidence of clinical malaria in this population. As expected, the risk of malaria-attributable morbidity increased with parasitemia but more importantly, at given levels of parasitemia, this morbidity risk also increased with age. Thus, in addition to demonstrating the likely acquisition of anti-parasitic immunity with age in this population, these data suggested that tolerance of parasitemia declines with age. It is not possible to conclude with certainty that a threshold effect of parasitemia on risk
of fever would have been calculable from this data as the sensitivity, specificity and predictive values of various cut-offs were not presented. This same method was applied to analyse data from a case (fever = 37.5°C)-control study of children 1-5 years recruited by household survey in the Gambia, in which the malaria attributable fraction in febrile cases was 44% and the sensitivity and specificity of a cut-off of 5000 parasites/µL was approximately 90% [27]. The same authors had previously found that a cut-off of 5000 parasites/µL maximised sensitivity and specificity (approximately 80% each) for the diagnosis of fever (axillary temperature = 37.5°C) in a cross-sectional household survey of children = 5 years in Tanzania [26].

Christophe Rogier and colleagues [190] used comparable mathematical modelling techniques to investigate the relationship between parasitemia and fever risk in Senegalese inhabitants of a holoendemic region. A cohort of 200 children and adults aged 1 month to 83 years were seen daily in their homes by a member of the study team and followed longitudinally for 4 months, with thrice weekly collection of clinical data (including temperatures) and bi-weekly blood smears for microscopic examination. Additional smears were taken and clinical examinations were conducted in all subjects with illness or fever (axillary temperature = 38°C or rectal temperature = 38.5°C). Controls had a temperature below these levels and no history of fever in the preceding 72 hours. The relationship between parasite density and risk of fever was modelled as a continuous function using the same approach as that used previously in PNG [25]. The overall malaria attributable fraction in febrile subjects from this population was 44%. Analogous to the PNG model, for the same parasite density, the risk of fever increased with age but the best tested continuous function model failed goodness of fit tests. The authors next tested models that examined a discontinuous relationship between parasitemia and fever risk by including an independent binary variable corresponding to a threshold value (i.e., the value of this variable was 0 if parasitemia was below the cut-off and 1 if above this cut-off). Goodness of fit was only slightly improved over the continuous function model when a single cut-off was used to define this variable irrespective of age or when multiple different variables were used for different age groups.

The model was significantly improved (and passed goodness of fit tests) when this pyrogenic threshold variable was defined as an inverse exponential function of age
(i.e., decaying rapidly from a maximum in children aged 12-23 months and then approaching a limit with extremes of age). This inverse exponential function paralleled the decay in parasite densities in asymptomatic individuals. In practice this meant that when an individual’s parasitemia crossed the threshold corresponding to their age (Figure 4.1), the subject-specific fever risk was multiplied by an odds ratio of 44 (95% CI 14-145; Figure 4.2). Importantly, variation in the threshold level between individuals of the same age was determined to be statistically insignificant. Results were similar when a different cut-off was used to define fever ($\geq 37.7^\circ\text{C}$ axillary; $= 38.2^\circ\text{C}$ rectal) and when either the maximum or minimum parasitemias during fever episodes were used in the model. Only 14 (0.3%) of 4936 measures of parasitemia in asymptomatic subjects exceeded the age-dependent pyrogenic threshold. Of 348 observations of parasitemia higher than the threshold made in 8636 observations from 247 inhabitants of the village in an “intention to test” analysis: 43% had a concurrent fever ($= 38^\circ\text{C}$ axillary); 65% had a fever during the same pathologic episode and 90% had fever or fever-related symptoms (20 of the remaining 36 observations were made in subjects who left the study). Hence, it appears from this data that even on an intention to test analysis, for the clinical diagnosis of malaria the age-specific pyrogenic threshold had a sensitivity of 90%; specificity of 99.7%; positive predictive value of 95.7% and negative predictive value of 99%. The results can be summarised by saying that in this population, using data defined in this way, there was “almost no overlap between parasitemias observed during malaria attacks and asymptomatic parasitemias” [190]. It is notable that the interaction between age and parasitemia in Senegal and PNG [25] was comparable, and that fever risk as a function of parasitemia increased similarly when modelled as a continuous function in both locations. This was evident from the exponents of the power function in the different models, which were very similar in PNG and Senegal (0.84 and 0.82 respectively) and also in a parallel study conducted in Tanzania (0.83; [26]).
Figure 4.1. The age-dependence of the pyrogenic threshold

“When a person’s parasitemia crossed the threshold corresponding to his or her age (years), the subject-specific fever risk was multiplied by an odds ratio of 44.4 (95% CI 13.6-144.8). Irrespective of crossing the threshold, an increase of 0.5 in the parasite/leucocyte ratio was associated with a multiplication of fever risk by 1.35 (95% CI 1.34-1.36)” [190]. It is notable that the differences between children and adults would have been exacerbated if parasitemia/µL was estimated from subject specific leucocyte counts, which decreased from 12800/µL in infants to 5400/µL in adults more than 60 years of age. Figure reproduced with the kind permission of Christophe Rogier.
Figure 4.2. Estimated subject-specific odds ratio of fever by parasitemia and age

Figure reproduced with the kind permission of Christophe Rogier.

The same methods [26,190] were used to examine data from a longitudinal study in the Madagascar highlands conducted from 1993 to 1996 following a malaria epidemic between 1986 and 1990 that killed 30000-40000 people [199]. Malaria transmission during the study period was low and seasonal (peak parasitemia rate in asymptomatic individuals was approximately 15% at age 3-4 years and declined with age to < 5% in those > 40 years). Subjects were aged 0-95 years (n=829; median age 14.3); cases with febrile illnesses (axillary temperature = 37.5°C) were recruited at a health centre and also detected by thrice weekly household visits; and asymptomatic controls were recruited during systematic monthly household visits. The overall malaria attributable fraction [23] in cases was 30% and showed little variation with age. Risk of fever increased with parasitemia (grouped 0; 1-499; 500-4999; 5000-49999; = 50000 parasites/µL) and there was only a slight and inconsistent increase in fever risk at given levels of parasitemia with age. Season and year were also significant predictors of fever risk. Neither a binary pyrogenic threshold variable nor an exponentially decaying pyrogenic threshold with age improved the basic model. The sensitivity for the best model for predicting fever was 25%, with a specificity of 97%. In further contrast to the results from Senegal [190], there was a significant individual random effect in the model, suggesting that natural immunity in the
Madagascan population was much more heterogenous than in Senegal and that the best logistic regression model had limited utility in the diagnosis of malaria at the subject-specific level. In summary, in this low transmission area, although the parasite density with fever decreased with age (similar to the relationship shown by Cox et al [13]) and the risk of fever at a given parasite density increased with age (as in Senegal [190]), these relationships were much weaker than those demonstrated in the high transmission areas; there was significant individual heterogeneity; and fever risk was likely to have been heavily influenced by seasonal and temporal fluctuation in transmission intensity.

Prybylski and colleagues [200] examined the relationship between parasitemia and fever/other clinical features in the Pakistani Punjab province where malaria endemicity is low and transmission is highly seasonal. Data were collected for almost 9000 patient encounters in outpatient clinics conducted approximately fortnightly over 13 consecutive months in 4 villages. The method of recording body temperature was not stated but fever was defined as a temperature of $\geq 38^\circ$C. Malaria attributable fractions were calculated by classical methods and multiple logistic regression modelling [23,26], excluding data from mixed species infections. Notably, parasite prevalence and density data in controls was obtained from clinic attendees rather than community controls and there was significant variation in parasite prevalence and density among different villages. Overall malaria attributable morbidity was 22% for \textit{P. falciparum} and 12% for \textit{P. vivax} (clinical illness), and 19% and 9% respectively for fever. The most important finding was a strong linear relationship between parasite density (categorised as zero, 1-999, 1000-4999 and $\geq 5000/\mu$L) and risk of both clinical malaria diagnosis and measured fever in subjects with \textit{P. falciparum} and with \textit{P. vivax}. This contrasts with the relationship between parasitemia and fever risk in highly endemic areas (see Figure 2.3) in which the risk of fever is low at parasitemias below approximately 10000 parasites/\mu L [25,190]. The proportion of clinical illnesses and fevers attributable to malaria in the Punjab increased with parasite density in the case of \textit{P. falciparum} but was much lower and relatively constant for \textit{P. vivax}. While significant trends for fever risk and attributable fraction were seen in each age strata for \textit{P. falciparum} ($< 4$, 4-12 and $\geq 13$ years), the magnitude of increased risk across parasite densities was lowest for children aged $< 4$ years. The overall malaria attributable fraction estimate (i.e., the
chance of illness or fever being due to malaria) increased with age in contrast to the negative trend demonstrated in the PNG and Tanzanian populations exposed to high transmission [24,25]. This is likely to reflect differences in age-acquired natural immunity, although differences in study design or the age-related epidemiology of other infections may also be partly responsible.

4.2.3. Resistance to the clinical effects of malaria

Based on the observation in endemic areas that, “severe disease and death from malaria declines with increasing host age much faster than the host’s ability to regulate fever, parasite densities and ultimately infection per se”, Snow and Marsh [14] have proposed three definitions of immunity anti-parasitic, anti-fever (i.e., tolerance) and anti-severe disease. While the mechanisms mediating the latter are poorly understood, it is reasonable to assume that they involve resistance to known determinants of severe disease including cytoadherence, toxin induced cytokine production and anemia [11]. In an attempt to separate the effects of host age from exposure to malaria on the acquisition of anti-malarial immunity, Baird and his colleagues have studied the early impact of malaria on transmigrant Indonesians migrating from an area of extremely low malaria endemicity (mostly Java) to a region of intense transmission (Papua province). Although interpretation of these studies is partly confounded by the administration of chloroquine prophylaxis to new arrivals in Papua and possibly differences in treatment seeking behaviour for individuals of different ages, a number of interesting observations have been made. Within the first 6 months of arrival in Papua, the evacuation rate for adults to the hospital because of severe malaria was 23% compared to 4% in children aged 6-10 and 10% in younger children [201]. Evacuation rates for children and adults were comparable in the succeeding 18 months. The authors reviewed a number of other studies of epidemic malaria supporting their view that children may be less likely than adults to suffer severe disease following primary exposure. Within 8 months of arrival, the typical age-related pattern of diminishing frequency and density of parasitemia characteristic of indigenous inhabitants of the region had become established, suggesting that constitutional differences related to aging rather than exposure were responsible [202]. Another study showed similar prevalence of *P. falciparum* and *P. vivax* parasitemias in children and adults after 14 months but
higher densities in the children [203]. Unfortunately, data on the rates and severity of uncomplicated disease in this unique population do not appear to be available.

It has been rightly noted that, “clinical descriptive studies of non-severe malaria attacks undertaken at the population level are rare” [204]. The initial severity of uncomplicated malaria attacks in children and adults living under conditions of intense transmission has been shown to be similar at all ages and not predictive of the overall duration of disease [204]. Although Cox and colleagues [13] were able to classify uncomplicated malaria into mild and very mild forms in PNG on the basis of the perceived need for treatment by the health services, clinic attendees had a significantly higher parasitemia which argues for differences in anti-parasitic rather than anti-toxic immunity. Karunaweera and colleagues [205] enrolled age-matched adult cohorts living in a non-endemic area (Colombo) and a region of low to moderate endemicity (Kataragama) in Sri Lanka and compared outcomes of *P. vivax* malaria. Patients from the malaria-endemic region had lower parasite densities at the time of presentation and lower plasma levels of TNF-a during symptomatic infections than their urban counterparts. The urban patients had significantly higher summed clinical scores (i.e., the sum of 11 malaria symptoms scored from 0 to 3) than their rural counterparts (19 vs 8.8 respectively). Of note, patients from both groups were of similar socioeconomic status and potential bias in reporting of symptoms was controlled for by rating clinical scores relative to previous febrile illnesses as an internal control. The significant difference in clinical scores persisted after controlling for the differences between the groups in parasite densities and plasma TNF-a levels, suggesting that endemic area patients were more tolerant of both parasitemia and the effects of TNF-a during illness. In another study, these authors reported that the ratio of clinical score to parasitemia was reduced in successive *P. vivax* infections (and also in a *P. falciparum* infection following successive *P. vivax* infections) but little changed by successive *P. falciparum* infections [206]. The ratio of clinical score to parasitemia showed an inverse relationship with increasing age, consistent with an innate age-dependent change given that a mild degree of anti-parasitic immunity appeared to develop during the study which increased with age. Unfortunately though, this was primarily an observational study and the statistical significance of its results was not presented.
4.2.4. Summary

It is important to note that the primary purpose of observational studies made in endemic areas was generally to evaluate diagnostic criteria for uncomplicated malaria attacks (with an emphasis on the relationship between parasitemia and disease) or to describe/estimate malaria morbidity. Significant methodological irregularities in some of these studies raise questions regarding the validity of their conclusions regarding pyrogenic thresholds and lack of standardisation in study design makes it all but impossible to compare the results of one study with another. These methodological differences include factors related to: subject age ranges; method of temperature measurement and definition of fever; timing of parasite density estimation in relation to measurement of temperature or recording of symptoms; calculation of parasitemia from blood smear examination; definition and precision of presumptive malaria cases (e.g., symptoms versus signs; temporal relationship to examination; experience of examiner); method of recruitment (household survey versus clinic presentation with illness); period of recruitment (duration of time and influence of seasons); statistical assessment of parasitemia (mean versus GM) and multiple observations from the same subject; and even potential financial inducement of researchers [189].

As these observational studies in general confirmed the existence of a monotonic relationship between parasitemia and risk of disease, it was entirely logical to evaluate the utility of different pyrogenic thresholds as part of the case definition for malaria. The consistent theme that emerged was that significant overlap existed between the parasitemias measured in presumptive malaria cases and controls, thus limiting the absolute discriminatory power of discrete cut-offs in terms of their positive and negative predictive values. Because of the methodological issues outlined above, it is impossible to know whether the “blurred” pyrogenic thresholds that resulted from parasitemia overlap could have been more precisely defined in these studies. Seasonal and temporal changes in malaria epidemiology (and attendant fluctuation in anti-malarial immunity) may also have influenced the relationship between parasitemia and fever such that even the conclusions of the better designed studies may be altered by the passage of time. Nevertheless, the possibility that pyrogenic thresholds may exist and decrease with age was raised in
observational studies, which prompted further research specifically designed to test these hypotheses.

It is only recently that research questions regarding the acquisition of anti-toxic immune responses in adults with deliberately induced malaria infections have been addressed [197,198]. Earlier reports tended to be primarily descriptive in nature and have concentrated on predominantly anti-parasitic responses with more attention paid to peak parasitemias during infection than on threshold levels of parasitemia associated with illness [207,208]. It is apparent that in previously malaria naïve adults, an innate pyrogenic threshold exists which varies according to the strain of the parasite and the genetics of the host, with those of African descent appearing to be more tolerant than Caucasians. Acquisition of immunity that results in an elevation of this threshold appears to develop very rapidly, possibly during the initial infection and more certainly by the time of a second infection. It is possible that more detailed examination of this data over time may enable other important questions to be addressed such as whether one *Plasmodium* species induces anti-toxic immunity to the other (extending the 1938 analyses of Boyd and Kitchen [207]) as well as further exploration of inter-strain differences. It is unlikely though that this database will yield answers to questions relating to the age dependence of tolerance and it is important to realise the methodological limitations of analysing data that was either not collected in a research setting or recorded for reasons other than the examination of anti-toxic immune responses. Hence, the results should be interpreted cautiously.

The results of studies that have been designed to specifically address the relationship between parasitemia and fever in regions of differing malaria endemicity are likely to be more precise in their conclusions than observational studies conducted for other reasons. In regions of high endemicity, 3 studies have demonstrated that pyrogenic thresholds of high sensitivity and specificity can be derived by mathematical modelling of data collected from cases and controls [26,27,190]. The most appropriate cut-off in 2 separate populations of children = 5 years from Tanzania [26] and The Gambia [27] was approximately 5000 parasites/µL. Given that the age-dependent pyrogenic threshold in early childhood appeared to fall very sharply in Senegalese children [190], it is possible that an exponentially decaying threshold
with age may have been of even higher sensitivity and specificity in the Tanzanian and Gambian studies. The pyrogenic threshold in children = 5 years in Senegal was much higher than 5000 parasites/µL, which may have reflected differences in malarial epidemiology; clinical case definitions; calculation of parasitemia; or the fact that higher temperatures were used for the definition of fever in the Senegalese study than in the other locations.

The precision of the pyrogenic threshold in the Senegalese study [190] was remarkable – it effectively divided malaria cases from asymptomatic controls on the basis of only one variable (parasitemia). While this may indicate that stably high malarial endemicity induces the same amount of tolerance across the population and be reflective of the methodological design of this study (which included intensive active case detection, rigorous definition of controls and the ability to detect short duration illnesses), this finding requires validation in follow-up studies. Two studies provided very good data to show that the pyrogenic threshold decreased with advancing age [26,190]. It is notable that the differences between early childhood and adulthood shown in the Senegalese study would have been further exacerbated had age-specific leucocyte counts been used in the calculation of parasitemias [190].

In regions of lower endemicity, the relationship between parasitemia and risk of malarial illness appears to be much less discrete than in regions of high endemicity, which is reflected by the poor fit of models with discontinuous pyrogenic threshold variables to the observed data. In the Pakistani Punjab, a region of very low endemicity, the relationship between parasitemia and fever risk was essentially linear for both *P. falciparum* and *P. vivax*, with clinical malaria occurring at even the very lowest parasitemias [200]. The proportion of febrile morbidity attributable to malaria increased with age, suggesting that acquisition of anti-malarial immunity does not show the same association with age evident in highly endemic areas. In Madagascar, a region of intermediate endemicity between Pakistan and the studies described above, it is likely that heterogeneity in the acquisition of malarial immunity related to season and year of enrollment affected the relationship between parasitemia and fever [199]. Malaria-attributable morbidity showed little change with age in contrast to the decline with age typical of highly endemic regions. Although the risk of fever increased with parasitemia, it was impossible to fit a
model with a pyrogenic threshold variable and there was only a weak association to suggest that the risk of malarial morbidity at a given parasitemia increases with age.

Thus it appears that as malaria transmission increases in a population, so does both the level of the pyrogenic threshold for clinical malaria and its precision. It seems likely that increased levels of anti-toxic immunity (manifested as “tolerance”) parallel the increase in pyrogenic threshold with endemicity, and that homogeneity in immune responses secondary to the stability of malaria transmission increases its precision. *Higher levels of asymptomatic parasitemia in children from highly endemic areas imply more efficient tolerance than adults, just as the decrease in parasitemia with age is consistent with the acquisition of anti-parasitic immunity.* It is possible that the same mechanisms mediating tolerance are responsible for the apparent initial resistance of previously malaria-naïve children to severe disease compared to adults [201] (suggesting that tolerance may in part be innately age-related) and the differences in severity of illness between Sri Lankan adults with *P. vivax* [205] (suggesting an acquired component from cumulative exposure). On the other hand though, the similar initial severity of illness in subjects of all ages from Senegal [204] and the very basic observation that, subsequent to the decay of passive maternal immunity, very young children are *least* able to limit the severity of illness in endemic regions [14] introduces a paradox that suggests another component of anti-toxic immunity may be in operation that *increases* with age.

### 4.3. Arguments against the existence and nature of anti-toxic immunity to malaria

Ever since anti-toxic immunity was first mooted as a separate component of anti-malarial immunity there have been doubts raised as to whether the inferences drawn from the epidemiological studies reviewed in the previous section are sustainable. As an example, Taliaferro writing on “Immunity to the malaria infections” in the textbook *Malariology* in 1949 argued that, in the absence of a proven malaria toxin, anti-parasitic factors might “produce a lowered parasite metabolism” [209]. As evidence has since accumulated to support a toxic basis for common malarial disease manifestations such as fever (Chapter 3.1), factors lowering parasite metabolism that prevent induction of fever could reasonably be considered anti-toxic in nature. The
point that Taliaferro raises is valid though – until it can be demonstrated that administration of a structurally identical molecule to a proposed malaria toxin can reproduce signs of toxicity in humans, or alternatively that a specific anti-toxin therapy can inhibit toxicity in humans, the very existence of immunity that is anti-toxic in nature is open to question.

4.3.1. Assumptions relating to the relationship between fever and parasitemia

The concept of the existence of a pyrogenic threshold is founded on the following assumptions made during investigative studies: that the fever measured is due to malaria; that a direct relationship exists between the parasitemia measured and the onset of fever; and that the peripheral parasitemia measured is reflective of the number of bursting schizonts. Notwithstanding difficulties with case definitions in endemic areas, the general observation that fever risk increases with parasitemia is a useful first step in attributing the presence of fever to malaria. That parasitemia levels in presumptive malaria cases are on the whole higher than in asymptomatic controls, even if the prevalence doesn’t differ much in regions of very high malaria endemicity, supports this view. It is very unlikely that non-malarial fevers could confound this interpretation by allowing malaria parasitemia to increase during non-malarial illness, in fact that evidence which is available suggests exactly the opposite [26,28]. This general relationship between parasitemia and fever has been further refined mathematically by use of attributable fraction estimation, which provides population estimates of the burden of morbidity that would be avoided in the absence of malaria. This potential problem does not exist in naïve subjects and is less of an issue in areas of low endemicity, where in general evidence for the acquisition of anti-toxic immunity has supported that from highly endemic areas.

It is not clear what the optimal timing is for measurement of peripheral parasitemia in relation to the best correlate with schizont rupture and the onset of fever. Jeffrey and colleagues [170] compared mean parasite densities (without reporting GM or median densities unfortunately) on the day prior to first fever following induced infection to the density on the day of first fever. Parasitemia increased over these consecutive days from a mean of 2055/µL to 10589/µL for the Panama strain of *P. falciparum*. Although it is not clear which measurement correlates best with the
onset of fever, arithmetic means are likely to be a very inaccurate representation of central tendency in this instance due to the highly skewed distribution of parasitemias typical of most studies [197]. In a detailed descriptive study, Delley and colleagues [20] measured parasitemias and temperatures thrice daily at 6 hour intervals in approximately 40 adults from Mali over a 12 day period in the dry season and again during the wet season. Twenty-four of these subjects were enrolled in both seasons and parasitemia were detected at least once in 80% of cases. The parasite densities associated with concurrent fever episodes varied widely, and in 15 of 16 cases no temporal relationship could be established between peaks in parasitemia and onset of fever. The authors strongly rejected any notion of a constant pyrogenic threshold [190] in this population of adults, although it must be noted that with small numbers and without a gold standard for attributing fever to malaria, this conclusion may not be definitive. Systematic errors in the calculation of pyrogenic thresholds within studies may potentially be minimised by large study numbers, a consistent definition of fever and an appropriate and fixed interval between onset of the fever and measurement of parasitemia. These conditions were most closely approximated in the study by Rogier et al [190], which also demonstrated that different definitions of fever or use of minimal or maximal parasitemias during fever episodes had little bearing on results.

4.3.2. Assumptions relating to the relationship between peripheral and sequestered parasitemia

4.3.2.1. The possibility that anti-toxic immunity is really incomplete anti-parasitic immunity

As malarial fever appears to result from the host inflammatory response to toxins released around the time that a schizont ruptures, the number of schizonts bursting just prior to the onset of fever is likely to be a better indicator of threshold parasitemia than a peripheral measurement. In the absence of sequential organ biopsy data or reliable “correlates with non-sequestering by-products of infection or substances that respond in a predictable way to overall parasite load” [210], it is impossible to accurately define the number of sequestering *P. falciparum* schizonts at any time during an infection. Mathematical modelling of intra-host dynamics of
*P. falciparum* infection has been attempted, but in reviewing the various proposed models [211], the authors were left “rather dissatisfied with their lack of realism” [212]. The threshold level of schizontemia is potentially estimable from blood smears taken just prior to fever onset in the case of *P. vivax* infection, but to date no studies have used mathematical modelling to estimate threshold densities for *P. vivax*. This would be a worthwhile approach given the suggestion of age-dependence in *P. vivax* pyrogenic thresholds in the study from the Solomons [194]. Together, these observations raise the central question underlying interpretation of pyrogenic threshold densities – how well does the peripheral parasitemia parallel schizontemia/toxin production, and what could influence this relationship? The null hypothesis would state that all humans, regardless of age, race or malaria exposure, have the same innate level of resistance to malaria and that fever would occur at the same critical level of schizontemia coincident with schizont rupture for a given parasite strain (acknowledging that different strains may have different threshold levels).

It has been hypothesised by Vounatsou et al [213] that an age-dependant decrease in the ratio of circulating to sequestered parasites (termed the “CS ratio”) could explain the “apparent tolerance” defined in two of the studies [25,190] reviewed in the previous section. This alternative hypothesis has been summarised as follows, “If the immune response to merozoites becomes more effective, this lessens the number of trophozoites arising from any given load of sequestered parasites. This in turn will appear to reduce the pyrogenic threshold estimated from peripheral parasitemia data. On the other hand, if the host response selectively attacks antigens involved in cytoadhesion, this will reduce the proportion of trophozoites which sequester, and hence increase the CS ratio and the apparent pyrogenic threshold” [213]. Although it can be supposed from the age-related epidemiology of severe malaria in highly endemic areas that anti-cytoadherence responses are acquired very early in life [214], there is no reason to believe they are lost, therefore the last statement of this hypothesis can be dismissed as not having any bearing on a changing CS ratio. Given that the ratio of peripheral pyrogenic thresholds in the Senegalese study in subjects of 1 year compared to 60 years (after adjusting for white cell counts of 12800/µL at 1 year and 5400/µL at 60 years [190]) was 12 to 1, it would be necessary for these youngest children to have the most effective anti-parasitised-
erythrocyte immunity to prevent their lack of anti-merozoite-invasion immunity leading to exponential expansion of parasitemia.

There is currently little evidence that killing/removal of parasitised erythrocytes peaks in early childhood and declines with age, even though this has been suggested by some as another possible explanation for the apparent age-dependence of tolerance [215]. Furthermore, both children (excluding infants) and adults from highly endemic regions appear to maintain chronic infections at reasonably stable peripheral densities for much of the time despite frequent turnover of individual parasite genotypes and fluctuation of synchronous infections [39,173,174] and the incidence of symptomatic infections at all ages is low [13,190]. This implies that, during these periods, children are just as effective at limiting exponential expansion of parasitemia as adults. Presumably this is because their repertoire of anti-parasitic responses is sufficient to recognise the diversity of merozoite and erythrocyte surface antigens present in their resident parasite population at the time [174]. This is not to say that the overall anti-parasitic repertoire is as broad in children as in adults, as children suffer more frequent malaria attacks than adults [13,25,190] and it is now well established that such attacks usually result from infection with a parasite strain to which the host lacks effective anti-parasitic immunity [216-218]. The hypothesis of Vounatsou et al has also been considered, and rejected, by others [198] on the basis that a degree of tolerance was observed to develop after only one prior infection [197,198], which was thought to be too soon for merozoite invasion to be significantly inhibited. Hence it appears that this hypothesis is unlikely to either explain the “apparent” existence nor the age-dependence of pyrogenic thresholds.

4.3.2.2. The effect of synchronisation and duration of infection

Other factors may potentially alter the CS ratio which could undermine the interpretation that pyrogenic thresholds decrease with age if these factors are unequally distributed in children and adults from endemic regions. Mathematical modelling suggests that the proportion of sequestered parasites may vary according to the degree of synchronicity and age of the parasite population, the level of fever and the effects of drug treatment [210,219]. Large fluctuations in peripheral parasitemia may have also been observed over a short period of time, frequently by a
factor of over 100 in a 6 hour period and occasionally by a factor of over 1000 [20], suggesting that release of sequestered parasites en masse may occur even in asymptomatic individuals. It is also possible that different parasite strains may vary with regard to their propensity to sequester, which was proposed as a possible explanation for the strain differences in pyrogenic thresholds noted by Gatton and Cheng [197]. None of these factors (other than drug use) were specifically controlled for in any of the epidemiological studies that have been cited above, therefore it remains uncertain to what extent these issues may have influenced the calculation of pyrogenic thresholds.

Synchronisation and longevity of parasite sub-populations has been studied longitudinally at the microscopic and at the molecular level by genotyping parasite sub-populations in individuals from Africa and PNG. A longitudinal study in Papua New Guinean children and adults showed longer durations of microscopically determined asymptomatic parasitemia in 4 year old children compared to older children and adults [22]. Periodicity was demonstrated for the dynamics of *P. falciparum* parasitemia in children aged 4-14 years (a paucity of positive blood smears meant this couldn’t be modelled in adults). In a follow-up molecular study, synchronous replication of individual genotypes was evident, however, despite the overall microscopic evidence for synchronicity there was no evidence for synchronous replication of different co-infecting genotypes [220]. The duration of individual genotype infections for *P. falciparum* was significantly longer for 4 year olds than for 5-14 year olds [220], in contrast to the positive relationship between duration of infection and age reported in Tanzania [174,221]. It is possible that differences in malaria epidemiology, such as co-infection with *P. vivax* in PNG which may lead to density dependent regulation and failed PCR-detection of *P. falciparum* [173], or study design may in part account for these differences. On the other hand, Bruce et al [173] believe the Tanzanian data [221] to be consistent with their own, only the authors’ interpretation of it to be wrong [174].

### 4.3.3. Inconsistent results from epidemiological studies

Finally, it is important to note that not all investigators have reached the same conclusions from epidemiological studies exploring the existence and nature of anti-
toxic immunity. Petersen and colleagues [222] measured body temperatures and examined blood smears on a single occasion in 1622 children and adults (aged from 1 to > 45 years) “living under conditions of continuous malarial transmission in Liberia”. Data were obtained from either 1986, 1987 or 1990, and in both the dry and rainy seasons. Subjects were excluded if acute non-malarial infections were diagnosable at enrollment. Age-specific mean body temperatures at parasite isodensity declined with age and after the age of 5 years, a temperature of 38-38.4ºC was said not to “identify subjects with parasitemia better than did a lower body temperature” [222]. The authors interpreted their data as not supporting the hypothesis that, “a special ‘anti-disease’ immunity exists independently of parasitologic immunity” [222].

The data presented can, however, be interpreted differently. Although the absolute number of subjects who were febrile (i.e., temperature = 38ºC) was not presented, it appears that in febrile subjects, the GM parasitemia declined from a maximum at age < 2 years to its nadir between ages 15-24 years. This negative correlation between age and parasite density parallels that described by Cox et al [13] in self-reporting febrile cases from PNG and is consistent with the general concept that the pyrogenic threshold decreases with age. It is unclear what effect sampling subjects at three different time-points in different seasons may have on the consistency of the data presented in the Petersen study, or if subjects with non-malarial fevers were accurately identified. The use of body temperature on a continuous scale as an indicator of malarial morbidity is also questionable and no account was made for physiological changes in body temperature with age in this study. Hence the certainty of the authors’ conclusions is open to question.

4.4. Conclusions

Evidence has accumulated to support the supposition first made over 60 years ago that a distinct component of anti-malarial immunity known as “tolerance” can be acquired following exposure to malaria which results in a raised threshold in the level of parasitemia required to initiate symptoms such as fever. The pattern of tolerance in a population appears to change with an increase from low/seasonal endemicity to high/stable endemicity in two important ways:
• The continuous relationship between fever risk and parasitemia becomes increasingly discontinuous and precise.
• Age-dependency of the pyrogenic threshold becomes increasingly obvious, with children as young as 1 year of age tolerating the highest densities of asymptomatic parasitemia in areas of highest endemicity, after which the threshold decays exponentially in early childhood before stabilising at a lower level in adulthood.

It is not clear whether the age-dependence of tolerance reflects innately-determined immune responses associated with age per se (for which the positive evidence is only circumstantial), or is influenced by factors associated with ongoing exposure to malaria and other antigens, or both. Although some have argued that a form of anti-parasitic immunity masquerades as tolerance, the reduction in disease incidence with age that parallels reduced parasite densities suggests that the age-dependence of tolerance and anti-parasitic immunity run in opposite directions. The speed with which tolerance can be acquired by previously naïve subjects and the limitation of exponential expansion of parasitemias by individuals of all ages in highly endemic areas for most of the time further argue against this hypothesis. On the other hand, little is known of the population dynamics of *P. falciparum* relating to sequestration and it is entirely plausible that systematic changes with age may confound the interpretation of the relevant studies discussed in this section. It seems unlikely though from the magnitude of parasitemias commonly observed in asymptomatic child residents of highly endemic areas compared to the pyrogenic threshold of previously naïve adults (noting that comparative data from children is unobtainable), and the parallel but limited observations from studies of *P. vivax*, that acquired tolerance simply does not exist. Of note, it appears that both host genetics and parasite strain differences may have some bearing on the relationship between parasitemia and fever risk.

In addition to tolerance, there is reason to believe that another form of anti-toxic immunity exists that may contribute to limiting disease severity but which does not share the same age dependence as tolerance. Although the mechanisms governing tolerance may also contribute to limiting disease severity to some extent, the separate
age-dependencies suggests that anti-toxic “anti-severe disease” immunity has other determinants. Difficulty in designing non-confounded studies to specifically address this question has meant that far less is known epidemiologically about this phenomenon than that of tolerance.
Chapter 5. Physiological basis of anti-toxic immunity to malaria

The physiological basis of anti-toxic immunity to malaria is even more unclear than its existence is uncertain. Theoretically, and given the present understanding of malarial pathophysiology, there are a number of potential ways in which anti-toxic immune responses may function. These include: inhibition of parasite metabolic pathways leading to reduced or defective toxin production (of which little is presently known); neutralisation of toxin at or shortly after the time of release that prevents the initial activation of the inflammatory cascade; interference with the cellular mechanisms triggered by toxin activation that lead to production of inflammatory mediators; and diminished host responsiveness to one or more of the inflammatory processes that follow. To some extent, these immune responses could be innately (and/or genetically) determined, as even first infections may result in different outcomes in different subjects infected with the same parasite strains [197,198]. Of more importance is likely to be the acquired components of such responses, either by augmentation of non-specific immunity through repeated exposure to malaria and/or other infections, or induction of specific anti-malarial immunity. A number of laboratory and field based observations have informed our understanding of the potential mechanisms underlying anti-toxic immunity, which have led to hypotheses worthy of testing in clinical studies. These will be discussed in the following sections.

5.1. Toxin neutralisation by antibodies

Antibodies to malaria toxin have been considered as a straightforward explanation of the age-dependent pyrogenic threshold described in highly malaria-endemic regions. The proposed mechanism is as follows (adapted from [190]): young children have the most poorly developed acquired anti-parasitic immunity, which results in the highest parasite densities; this parasitemia induces a proportionate T-cell independent anti-toxic IgM antibody response; clinical malaria results when the amount of toxin exceeds the amount of antibody (i.e., when anti-parasitic immunity is insufficient to control expansion of a previously un-recognised parasite strain); as parasite densities decrease with age, so does stimulation of anti-toxin antibody production, hence the
pyrogenic threshold is overcome at lower parasitemias as age increases. This immunity would be expected to be short lived given the general kinetics of an IgM antibody response [223].

5.1.1. *In vitro and in vivo* laboratory studies

The above explanation was founded on the general understanding of antibody responses and supported by laboratory data derived from studies on the characterisation of malaria toxin in the early to mid 1990s. Initially it was demonstrated that IgM antibodies were produced by immuno-competent and T-cell deficient mice in response to challenge with non-protein antigens that were derived from boiled supernatants taken from *in vitro* *P. falciparum* cultures [224,225]. Serum from the mice could be used to block toxin-induced TNF-a production *in vitro* and *in vivo* [224]. This blocking activity was acquired within 7 days of immunisation and did not seem to be enhanced by boosting [226] or common adjuvants [227]. Concern that some of these findings may have been confounded by *Mycoplasma* contamination of *P. falciparum* cultures [119] was partially allayed by follow-up studies showing that the inhibitory antibodies specifically recognised PIs [226] (GPIs are not found in *Mycoplasmas*, which lack a cell wall [228]). It was shown soon after that a monoclonal antibody recognising GPI neutralised the toxic activity of whole *P. falciparum* schizont extracts *in vitro*, thus narrowing the specificity of the response to the most likely candidate toxin [148]. In a similar experiment, the TNF-a inducing ability of geographically diverse parasite strains was shown to be inhibited by a single monoclonal antibody [229].

Subsequently it was shown that serum from mice immunised with supernatant derived from the serum of 3 patients with acute *P. vivax* infection exhibited blocking activity against *in vitro* TNF-a induction by exoantigens of *P. vivax*, *P. falciparum* and *P. yoelii* [156]. Conversely, mouse immune serum raised against *P. falciparum* and *P. yoelii* exoantigens also inhibited *in vitro* TNF-a induction by the *P. vivax* supernatants. Soon after, the same group determined that acute and convalescent serum from a patient with acute *P. falciparum* malaria and another with *P. vivax* inhibited TNF-a production by human monocytes stimulated with *P. falciparum* culture supernatant-derived antigens [157]. The inhibitory activity of serum from
both patients: could be demonstrated against geographically diverse strains from 3 different continents; was shown to be IgM dependent; and could be blocked by pre-treatment of the patient sera with PI-liposomes, suggesting that the toxins of *P. falciparum* and *P. vivax* may be structurally similar. The same group raised antiserum in mice vaccinated with a non-toxic PI compound (purchased commercially) that was conjugated to keyhole limpet cyanin (KLH) [230]. In contrast to the earlier results, blocking activity against *in vitro* TNF-a induction by *P. falciparum* supernatants was substantially boosted and “strikingly prolonged” by repeat injection of PI-KLH. In addition, this blocking activity was shown to predominantly depend on IgG rather than IgM.

In an important follow-up study, Schofield et al [147] demonstrated that immunisation of mice with chemically synthetic *P. falciparum* GPI glycan conjugated to KLH reduced the early mortality (= 12 days) of *P. berghei* from 100% to 25%. There was no significant difference in parasitemia levels between the two groups, however, and immunised rodents eventually succumbed to hemolytic anemia accompanied by massive parasitemia by day 15. The KLH-glycan induced IgG antibodies in immunised mice were shown by immunofluorescence to bind to intact intra-erythrocytic trophozoites and schizonts but not to uninfected erythrocytes (which express endogenous GPls on their surface). The antibodies also recognised GPI-modified proteins of various molecular weights on a Western blot analyses of material from *P. falciparum*-infected erythrocytes. Serum from immunised mice completely neutralised production of TNF-a by mouse macrophages in response to stimulation by crude *P. falciparum* schizont extracts, suggesting that GPI alone is both sufficient and necessary for the induction of this inflammatory response. These results suggest that malarial GPI is highly conserved across different species of *Plasmodium*; confirm the central importance of GPI to toxicity in mice; and, to the extent that the severe malaria syndrome induced by *P. berghei* in mice parallels that in humans [231], provides some support for an anti-toxic vaccination strategy.

### 5.1.2. The role of CD1 in antibody responses to GPI

The physiological basis of antigen processing leading to antibody formation may be different for GPls than for proteins. It has recently been shown that various types of
lipid and glycolipid molecules can be presented to T-cells by CD1 molecules, which are encoded by genes outside the major histocompatibility complex (MHC) [232]. CD1 molecules are expressed on the surface of antigen presenting cells (e.g., macrophages, dendritic cells and B-cells) and have been divided into four subtypes (CD1a through d) according to their antigen specificity [233]. Human CD1b has been shown to bind and present the mycobacterial GPIs, lipoarabinomannan (LAM) and PI mannoses, which have the same basic lipid anchor as *P. falciparum* GPIs [232]. In the presence of LAM and CD1+ antigen presenting cells, CD1b-restricted T-cells taken from the skin of a patient with leprosy have been shown to induce IgG1 and IgG3 subclass antibody production by B-cells at the expense of IgG4 and IgE [234]. Mouse CD1d (which is highly homologous to human CD1d [235]) binds GPI with high affinity in mice via the PI moiety [236] and has been shown to strongly stimulate immunoregulatory natural killer (NK) T-cells *in vitro* after binding a variety of purified phospholipids (including PI) [237].

Whether or not CD1d molecules recognise malarial GPIs is controversial and unresolved. It has been reported that CD1d recognition of GPI-anchored *P. falciparum* (sporozoite) surface proteins leads to NK T-cell stimulation and that CD1d-complexed *P. berghei* circumsporozoite (CS) antigen (thought to be GPI anchored) elicits NK T-cell help in regulating IgG production [238]. Others, in attempting to replicate these results using CD1d and MHC class II-deficient mice in alternate experiments, have demonstrated that the IgG response to the CS protein was solely MHC class II-dependent [239], in line with previous evidence [240]. Most recently, a group working independently of the other two again demonstrated that IgG responses against *P. berghei* CS antigen were unaffected in CD1d-deficient mice [241], which the authors stated “contrasts sharply with [the] recent report by Schofield et al [238]”. Genetic differences between the mice used in the three studies were thought not to explain the discrepancies in results [241]. Similarly conflicting results have emerged from recent studies examining the role of CD1d-stimulated NK T-cell regulation of antibody production in response to *Trypanosoma cruzi* GPIs [242,243], which share structural similarities with those of *P. falciparum* [140].
5.1.3. Human studies

Jakobsen and colleagues [244] measured the seroprevalence of precipitating antibodies (using an assay that did not discriminate between isotypes or subclasses) to a soluble antigen of *P. falciparum* (precipitated from culture supernatant by binding to IgG from semi-malaria-immune donors) in Gambian children and adults exposed to highly seasonal malaria transmission. This antigen had previously been demonstrated to induce TNF-α production in a human monocyte cell line and was pyrogenic in rabbits [245], however, it is uncertain whether *Mycoplasma* may have contaminated those cultures [119]. At the end of the transmission (rainy) season, 40% of 1-2 year old children had antibodies, with the prevalence increasing with age to adult values of > 80% by age 9-11 years. Antibody responses were assessed twice in a subset of 3-8 year old children, once during the dry season and again at the end of the rainy season (curative treatment was given to all children at the start of the rainy season). There was no correlation between antibody response during the dry season and subsequent likelihood of asymptomatic, mild (parasitemia < 5000/µL) or moderate (parasitemia = 5000/µL) malaria as detected by weekly morbidity surveys. The prevalence of children with a positive antibody response significantly fell, however, from 100% to less than 50% in children who did not appear to be infected during the rainy season, suggesting that without boosting these antibodies were short-lived.

The same group [246] measured antibodies to commercially obtained PI in 65 Gambian children with mild (febrile and parasitemia = 2500/µL; outpatient), moderate (hospitalised) and severe (according to WHO criteria) malaria. Anti-PI IgM levels were significantly higher in children with severe compared to mild malaria and intermediate in the moderate group, but there were no differences in anti-PI IgG levels. In a very similar experiment, Facer and Agiostratidou [247] measured anti-PI antibodies in East African children with uncomplicated and severe malaria and found exactly the opposite – that children with severe malaria had significantly lower levels of anti-PI IgM than those with uncomplicated malaria. TNF-α was also measured in serum but did not correlate with antibody levels. In a subsequent study, Jakobsen’s group demonstrated increased anti-PI IgG levels in children with clinical malaria compared to healthy controls [248].
Das et al [249] measured anti-phosphatidylcholine antibodies in Indian adults with malaria (using commercially obtained phosphatidylcholine) and found that IgG levels were highest in those with cerebral malaria, lowest in healthy controls and intermediate in subjects with uncomplicated malaria. There was no correlation between IgM levels and malarial severity. IgG₁ subclass antibodies (but not total IgG) were inversely correlated with serum TNF-a levels in patients with malaria (considered as a single group). The authors reported quantitative differences in IgG subclass levels but did not standardise their results against either known levels of antibody in the positive control or differing affinities of the secondary antibodies. Using a hemozoin preparation derived from *P. falciparum* and pre-treated with detergent and proteinase for detection, Biswas et al [132] showed that Indian adults with either *P. falciparum* or *P. vivax* malaria produced higher level IgG and IgM antibody responses than both endemic and non-endemic area controls. Sera rich in precipitated IgM from adults with *P. falciparum* malaria inhibited hemozoin-induced TNF-a induction in volunteer human PBMCs but no effect was seen with IgG nor with IgM from *P. vivax* patients.

Of note, despite the reported purification of *P. falciparum* GPIs as early as 1993 [124], there were no reports in the literature of anti-GPI antibody seroprevalence surveys in malaria-exposed populations until late 2000 [140] (discussed in the context of the study presented in Chapter 14).

5.2. Down-regulation of host inflammatory responses

5.2.1. Endotoxin tolerance as a model for malarial tolerance

The phenomenon of endotoxin tolerance was initially defined in the 1960s on the basis that rabbits could be effectively immunised with a low dose of bacterial endotoxin (LPS) against death from the subsequent injection of a lethal dose (reviewed by West and Heagy [250]). The tolerant state in animals was later shown to correlate with a diminution in TNF-a production in response to repeated endotoxin injection that persisted for several weeks [251]. Subsequently it was shown that LPS-stimulated production of TNF-a, IL-1β, IL-6 and IL-10 in whole blood taken
from human volunteers 3 hours after an intravenous injection of *Escherichia coli* LPS was significantly reduced compared to baseline but restored at 24 hours (before and after standardisation for monocyte counts) [252]. Further *in vitro* experiments using human and animal monocytes/macrophages defined the characteristic physiological changes accompanying repeated exposure to endotoxin: inhibition of TNF-α; augmentation of NO and PG-E₂; and variably altered IL-6, IL-1 and IL-8 secretion [250].

Remarkable progress had been made in understanding the molecular basis underlying the cell signalling pathways initiated by LPS in monocyctic cells (reviewed in detail by Dobrovolskaia and Vogel [253]), which has also facilitated a better understanding of the events mediating tolerance. On the other hand, separating the primary effects of endotoxin from that of released products acting in a paracrine or autocrine manner *in vitro*, as well the effects of mediators liberated in response to endotoxin from non-macrophages sources *in vivo*, has proved challenging [253]. There is general agreement that optimal responses to LPS derived from *Enterobacteriaceae* depend on recognition by toll-like receptor-4 (TLR-4) complexed with CD-14 and the membrane-associated molecule MD-2 [254]. Binding of LPS leads to induction of protein kinases, which in turn activate nuclear factors such as NF-κB [255]. Activated NF-κB typically comprises two protein subunits from the Rel family of transacting proteins: p50, which mediates DNA binding; and p65, which mediates DNA binding and the transcriptional activation of numerous inflammatory cytokine genes [253].

It is presently unclear to what extent endotoxin tolerance may represent blockade of the intracellular signalling events required for gene expression, down-regulation of LPS receptors on the surface of immune cells or over-production of anti-inflammatory cytokines such as IL-10, TGF-β and NO [253,256]. While there is data to support each of these hypotheses, the bulk of available evidence supports the first proposition – that endotoxin tolerance arises secondary to alteration in signal transduction pathways. Desensitised human monocytes rendered tolerant by pretreatment with LPS have been shown to accumulate an excess of functionally inactive NF-κB complexes comprised of p50 homodimers [255,257] as well as elevation of the NF-κB-inhibitory protein IκBα [258,259]. The activation of other
kinases in the signal transduction cascade have also been shown to be inhibited in tolerant cells [250].

Other bacterial and non-bacterial products, as well as endogenous stimuli resulting from trauma or surgery, may result in refractoriness to LPS challenge even though the pattern of cytokine stimulation may differ subtly to that induced by the LPS of Enterobacteriaceae [250]. Consistent with this observation is the identification of other TLRs such as TLR-2, which interacts with ligands such as lipoteichoic acid (LTA) from Gram-positive bacteria, LAM from Mycobacteria [260] and GPI from T. cruzi [261] to induce cellular functions with similar but different intensities to that of LPS. The induction of cross-tolerance independent of paracrine mediators by ligands interacting differentially with either TLR-2 or TLR-4 suggests that these two receptors are likely to share common intracellular signal transduction pathways [262]. In more detailed studies, it has been shown that T. cruzi GPI-mucin and E. coli LPS use functionally similar pathways to induce TNF-a and IL-12 production in murine macrophages and that the two molecules exhibit cross-tolerisation as evidenced by inhibition of TNF-a and IL-12 production in response to secondary stimuli [263].

The practical relevance of these studies to human malaria may have been hinted at as early as 1965. Rubenstein and colleagues [264] injected four prisoners with commercially prepared bacterial endotoxin (derived from Salmonella abortus equi) and then deliberately infected them with P. vivax. All four experienced a typical malarial illness and were given treatment after 2 to 3 weeks of illness, which was followed within a week by another dose of endotoxin (interval 21 days in 2 subjects and 35 days in the others). The febrile response to endotoxin (fever index; equal to the area under the 7-hour post-injection temperature curve) was reduced by 59%, 67% and 81% in 3 subjects but was unchanged in the other (interval 35 days). In 2 subjects given a 3rd dose of endotoxin 21 days later (42 days from baseline), the fever index returned to normal in one and made up half of the loss in the other. The phenomenon of clinical tolerance to multiple injections of bacterial endotoxin had been well characterised by this time and the intervals had been deliberately chosen so that tolerance to the first dose of endotoxin would have waned.
These results in prisoners were consistent with an earlier observation in neurosyphilis patients, in whom the febrile response to typhoid vaccine was reduced in post-malarial therapy patients (parasites unspecified) compared to normal controls [265]. Together these observations are consistent with the results of the above-mentioned \textit{in vivo} and \textit{ex vivo} studies in animals and humans and suggest that repetitive exposure of monocytes to malaria toxins may induce a tolerant state characterised by down-regulation of important inflammatory mediators such as TNF-α. The demonstration that monoclonal antibodies inhibited TNF-α induction by malaria parasites but not by bacterial LPS or LTA [229] further supports the notion that the cross-tolerance evident in prisoners and neurosyphilis patients was due to an innate non-specific mechanism as described above rather than the effect of antibodies. Of interest, this theory is not consistent with the observations of Karunaweera and colleagues [205] in respect to resistance against severity of symptoms in \textit{P. vivax}, as these authors found that levels of TNF-α were higher in semi-immune patients with similar level clinical scores to non-immune controls.

5.2.2. The role of nitric oxide in anti-toxic immune responses

The role of NO in human malaria has been a topic of considerable interest and debate since it was first raised by Ian Clark and colleagues in 1991 [266]. NO has been proposed as both the primary mediator of tolerance to human malaria in highly endemic regions [267] as well as a key determinant of resistance to severe disease in populations of varying endemicity [179]. The age-dependencies of these two epidemiological phenomena differ quite strikingly (Chapter 4.2.4) and there are no reliable animal models that exist to enable resolution of this paradox as it relates to NO production. Furthermore, there have been no studies to date in which NO has been administered to or inhibited in human subjects, hence despite a growing amount of circumstantial evidence its precise role is yet to be defined.

5.2.2.1. Postulated role of nitric oxide in diminution of malarial disease severity

\textit{In vivo} NO production has been measured in the context of human malaria by a variety of different methods including estimation of NOx in serum [96,102-105,268-279] and CSF [110,270,273]; and measurement of NOS2 protein [96], NOS2 mRNA
and NOS enzyme activity [97,280] in PBMCs. Direct comparison of the results from these studies is difficult because of significant methodological differences relating to case/control selection, clinical malaria definitions and measurement of NO production (reviewed in detail by Le vesque et al [281]). The major discrepancy in the interpretation of systemic NOx production was that few studies attempted to control for the potentially confounding effect of decreased kidney function on NOx levels [96,273,274,277,279]. Only one of these studies included non-affected controls and controlled for the important confounding effect of dietary ingestion of nitrates [96].

In contrast to the early suggestions from uncontrolled studies that severe malaria was associated with increased NO metabolites [103,105,269], the general theme to emerge from controlled studies of systemic NO production and PBMC NOS2 expression/activity is that NO and disease severity are inversely associated. In one of these studies, the peak age of cerebral malaria morbidity coincided with an age-related nadir in NO production [282]. Unfortunately, no studies have prospectively examined disease risk in relation to constitutionally determined NO production and data from adults has been less informative than from children. However, in one retrospective study it was shown that PBMC NOS activity after recovery was significantly lower in Gabonese child survivors of severe malaria than in controls with mild malaria [97]. This finding especially supports the concept that genetically determined NO production could influence disease outcome.

Indeed, the most direct evidence that NO production could diminish disease severity in humans has been provided by genetic association studies linking polymorphisms in the NOS2 gene with differences in disease outcome [283-286]. The advantage of disease association studies is that they may identify a role for particular disease mediators independent of other effector mechanisms. This can be useful in elucidating the importance of processes identified in simple (e.g., in vitro) models, while at the same time minimising the “noise” apparent in complex in vivo (e.g., human) systems. These studies must, however, be interpreted with extreme caution [287] as the potential for misinterpretation of results secondary to failure to consider the effect of multiple statistical comparisons may range from moderate to enormous and be difficult to discern from published reports. This may be one reason that
NOS2 polymorphisms thought to be important in one region are associated with negative (or sometimes, completely opposite [284,286]) results in others. In addition to confirmatory disease association studies in different settings, the case for universal relevance of NOS2 polymorphisms can be strengthened by studies demonstrating that the polymorphism in question is associated with functional differences in NO production [285]. The studies linking NOS2 polymorphisms with altered disease outcome and/or functionally different NO responses have been reviewed in detail in the context of the data presented in Chapter 13.

The cellular source(s) and regulation of NO production in the setting of asymptomatic and clinical malaria are incompletely understood, as are the mechanisms by which NO may retard disease pathogenesis. Systemic levels of the potent NOS2-inducing cytokines TNF-α, and IFN-γ are positively associated with the severity of clinical malaria [44,288,289], whereas IL-12 levels are inversely associated with severity [290-292]. As IL-12 is thought to induce NO predominantly via stimulation of IFN-γ [293,294], it is possible that both IL-12 and NO are inhibited by other mediators in the setting of severe malaria. One candidate down-regulatory molecule that is over-produced in severe malaria is IL-10 [96,288,290,291,295] and there is also indirect evidence to suggest that macrophage ingestion of malarial hemozoin may lead to down-regulation of NO production [130].

In the absence of prospective studies it is difficult to be certain whether the markedly diminished systemic NO production typical of severe malaria results from a near-complete genetic inability to produce NO in this setting, however, this explanation seems unlikely as PBMCs isolated from children who had recovered from severe malaria exhibited reasonably high levels of NOS activity (albeit less than children with past mild malaria [97]). Additionally, the positive immunohistochemical staining of NOS2 in brain tissue from cerebral malaria cases [107,109] suggests that NOS2 is expressed in some sites and more likely to be differentially regulated or stimulated in the brain and periphery rather than unresponsive to induction. The possible mechanisms by which NO could modulate the pathological events critical to malarial disease severity include down-regulation of inflammatory cytokine activity.
(see below and [296,297]) and inhibition of endothelial cell surface receptor expression. This is reviewed in detail elsewhere [179].

5.2.2.2. Postulated role of nitric oxide in tolerance of human malaria

NO has been postulated to mediate the age-dependent tolerance of chronic malaria infection in highly endemic regions via a mechanism akin to that of endotoxin tolerance [267]. The observation that asymptomatic child residents of both Tanzania and Madang, PNG produced more NO than their malaria-affected counterparts [96,272] was cited to support this hypothesis [267]. In contrast, levels of TNF-a in the Madang children were directly related to disease severity, with plasma NOx/TNF-a ratios highest in asymptomatic children and lowest in those with severe disease [272]. The plasma NOx levels of the asymptomatic Madang children were reportedly significantly higher than in adults from the same region, however, dietary ingestion of nitrates was not controlled for in these studies [267,272]. The age profile of NO production in the Tanzanian children showed that NOx levels were highest at age 1, reached a nadir at 5-6 years and then increased again to age 9 [282], but it is important to note that this population was exposed to a lower level of malaria transmission than is present in Madang [193]. The high levels of urinary and plasma NOx and universal PBMC NOS expression evident in asymptomatic malaria-exposed Tanzanian children [96] contrasts not only with the low levels in diseased children but also with the very low levels reported in US control adults [298,299].

The presence of *P. falciparum* parasitemia on microscopy and PCR in the asymptomatic Tanzanian children was associated with significantly higher NOx production than in microscopy/PCR negative children [282]. NOx levels in the thick film negative/PCR positive children were much closer to levels in aparasitemic children than in thick film/PCR positive children, which may suggest that *P. falciparum* parasitemia induces NO production once a threshold level is reached. More recently it was shown that voluntary infection of malaria-naïve healthy adults with *P. falciparum* merozoites was associated with strong induction of NOS activity in PBMCs after asymptomatic sub-microscopic/PCR positive parasitemia was allowed to develop [300]. The positive association between parasitemia and NO production in these studies is broadly in agreement with data from the majority of
animal studies (reviewed by Anstey and Weinberg [179]) but contrasts with observations in some in vitro and animal models associating NO with anti-parasitic responses [301,302]. Collectively, these results identify NO production by PBMCs (and possibly other cellular sources) as potentially down-regulating TNF-a secretion by mononuclear cells in an age-dependent manner in response to infection with *P. falciparum* (and possibly other stimuli). The potential stimuli and sources of NO production in tolerant individuals is reviewed and discussed in detail in the context of the data presented in Chapter 11.

The hypothesis outlined above is founded on the assumption that the augmented NO production observed in residents of malaria-endemic regions is not only reflective of the endotoxin tolerance-like state (in parallel with decreased TNF-a production) but that NO itself mediates the reduction in TNF-a output by mononuclear cells. The only experimental evidence supporting this latter statement has been derived from studies in animals, which in part reflects the widespread (if erroneous) belief that it is difficult to induce measurable NO production in human mononuclear cells in vitro [98]. Administration of the NO inhibitors \(N^G\)-nitro-L-arginine-methyl-ester or \(N^G\)-monomethyl-L-arginine (L-NMMA) to rats just prior to injection of a sub-lethal dose of LPS increases mortality to a second lethal dose of LPS given 24 hours later [303,304] and is associated with increased TNF-a production [304]. That this may be due to an autocrine effect of NO secreted by mononuclear cells was suggested by experiments showing that L-NMMA pre-treatment similarly disinhibited TNF-a secretion by murine macrophages in a dose-dependent manner in vitro [256]. Pre-incubation of cells with NO donors also reduced LPS-stimulated TNF-a secretion in this study. As the effect of NO on LPS-induced desensitisation were not evident under all experimental conditions, the authors were careful to stress that, “LPS tolerance involves more than [just] NO-mediated feedback” [256]. Very recently it was shown that LPS-induced endotoxin tolerance was not dependent on NO production in vivo, as similar reductions in TNF-a production to repeated injections of LPS were demonstrable in wild-type and NOS2-knockout mice [305]. This observation is consistent with the present understanding of the molecular basis of endotoxin tolerance [250].
Chapter 6. Hypotheses and aims of this work

The primary aim of this thesis was to investigate candidate mechanisms proposed to mediate anti-toxic immunity to malaria, with a particular emphasis on the processes that enable tolerance of chronic parasitemia. The focus of this thesis was restricted to the two mechanisms that have attracted the most research attention and for which evidence has accumulated for a potential role in anti-toxic immunity – NO production and anti-GPI antibody formation. In light of the evidence summarised in the previous chapters, the specific hypotheses to be addressed were:

- Basal systemic NO production shows the same age-dependence as tolerance in highly malaria endemic populations.
- Systemic NO production is triggered by, and proportional to, malaria parasitemia.
- Both *P. falciparum* and *P. vivax* trigger NO production.
- As NO production can be triggered by IgE and IL-12, both would be positively correlated with basal NO production.
- The main source of systemic NO production in highly malaria-endemic populations is circulating mononuclear cells.
- Genetic polymorphisms in the NOS2 gene and the genes of NOS2-inducing cytokines influence basal NO production.
- Residents of highly malaria-endemic populations make antibodies that recognise *P. falciparum* GPIs in response to infection with *P. falciparum* and also in response to infection with *P. vivax*.
- Anti-GPI antibodies show the same age dependence as tolerance.
- Anti-GPI antibodies are short-lived, with a predominance of IgM over IgG.
- Levels of anti-GPI antibodies are proportional to parasitemia, therefore children are expected to have higher antibody levels than adults.
- Alternatively, either or both of these proposed mechanisms could show an age-dependence that parallels resistance to severe manifestations of malaria (i.e., increasing with age rather than decreasing with age).
The aim of this thesis was to test these hypotheses experimentally in field and laboratory studies by enrolling asymptomatic residents of highly malaria-endemic regions who span a broad age range from childhood to adulthood. NO production was assessed by measuring plasma levels and urinary excretion of NOx, as well as PBMC NOS activity and antibodies were assayed that recognised purified *P. falciparum* GPIs. The association between parasitemia and NO production/anti-GPI antibody formation was tested cross-sectionally prior to any treatment and longitudinally after pharmacological eradication of parasitemia. Genetic determinants of NO production were investigated by genotyping subjects for NOS polymorphisms proposed to influence NO production and alter the risk of malaria severity, as well as polymorphisms in the IL-12 gene that may influence IL-12 expression and NOS induction.
SECTION Two.

General experimental procedures
Chapter 7. Ethical standards and approvals

All of the research detailed in this thesis was conducted in accordance with the Australian National Health and Medical Research Council’s *National Statement on Ethical Conduct in Research Involving Humans* [306], as well as conforming to the standards of the corresponding national and local authorities in Indonesia, PNG and Tanzania.

### 7.1. Indonesian study (Chapter 11)

The study protocol was separately approved by the Human Research Ethics Committee (HREC) of the NT Department of Health and Community Services and Menzies School of Health Research (MSHR; formerly the Joint Institutional Ethics Committee of the Royal Darwin Hospital and MSHR) and the Ethics Committee of the National Institute of Health Research and Development (NIHRD), Indonesian Ministry of Health and Social Welfare, Jakarta, Indonesia. Approval to conduct the study was also sought and obtained from the relevant provincial, regional and local administrative and health authorities within Papua province. Permission to work in each village was obtained from the respective village leaders after explanation of the study protocols and consent process.

Informed consent was obtained from individual participants within the guidelines specified by the HREC and National Health and Medical Research Council (NHMRC) [306] and consistent with local customs in Papua. All verbal and written information regarding the study and consent process was translated into Bahasa Indonesia. Signed written consent was obtained from individual study participants following verbal and written explanation at the time of screening and again at enrollment.

### 7.2. Papua New Guinea study (Chapter 12 to Chapter 15)

The study protocol for the Madang project was separately approved by the HRECs of the MSHR, the Northern Territory University and the PNG Medical Research
Advisory Committee. Approval to conduct the study was also obtained from the Madang Provincial Administration Health Branch. Permission to work in the study villages was sought and obtained through the well established process of consultation between researchers of the PNG Institute of Medical Research (PNGIMR) and representatives of the study villages and families known as “tok save”, literally to communicate and foster understanding.

Informed consent was given verbally by individual participants, as well their guardians in the case of children, and agreement was recorded on each individual’s enrollment forms through the signature of one of the investigators. This process of obtaining and recording individual consent was culturally appropriate and consistent with the guidelines outlined in the NHMRC *National Statement on Ethical Conduct in Research Involving Humans* [306].

### 7.3. Tanzanian study (Chapter 13)

Ethical approval for the study protocol had previously been obtained from the College Research and Publications Committee at Muhimbili Medical Centre, Tanzania and the Institutional Review Board at Duke University Medical Centre [96]. Informed consent was obtained from the parents and guardians of children in Kiswahili. Subsequent approval for genotyping the two IL-12 polymorphisms was obtained from the HRECs of the MSHR and the Walter and Eliza Hall Institute.
Chapter 8. Field protocols

The following procedures relate mainly to those used in the collection and initial processing of data, blood and urine samples at the Genyem (Papua) and Madang (PNG) field sites. As similar procedures were used in the recruitment of Darwin controls at MSHR, they are described here also.

8.1. Facilities and staff

8.1.1. Genyem Health Centre and US Naval Medical Research Unit-2 laboratory, Jayapura

Field work was based at the regional health centre in Genyem where 6 beds were provided to the project for overnight supervision of study participants. The Genyem Health Centre had previously been established as a field centre for other studies conducted under the MSHR-NIH RD research collaboration (see [307] for map). Laboratory facilities for preparation and microscopic examination of blood slides, venesec tion, separation of plasma by centrifugation and temporary storage of specimens at -20°C were available at the health centre. The medical director contributed to the field work part time and released two nurses and one laboratory technician to be wholly dedicated to the project. A four wheel drive vehicle was available for transport between the health centre and villages (all within 16 km radius). Transport of specimens between Genyem and the provincial capital Jayapura by dedicated taxi took 2 hours. Frozen samples were transported to Jayapura in a liquid nitrogen dry shipper.

The US Naval Medical Research Unit (NAMRU)-2 laboratory in Jayapura was modern and well-equipped and was available for use through an agreement between MSHR and the US Navy. Facilities included a Coulter counter, centrifuge, class II biohazard hood and -70°C freezers. A laboratory scientist was employed full-time by MSHR to work on this and related projects.
8.1.2. Madang, Papua New Guinea

Field work in Madang was based at the PNGIMR laboratory in Yagaum which was located approximately 35 km by road from the study villages and 16 km from Madang town. The Yagaum laboratory was modern and well-equipped with a centrifuge, class II biohazard hood, -20°C and -70°C freezers, and the facility to obtain liquid nitrogen. Two experienced field workers from the PNGIMR were dedicated to the project and a resident of one of the study villages was trained and employed to work part-time at the field sites. An additional expert microscopist with over 10 years experience was seconded from the PNGIMR to cross-check thick and thin blood smears. Transport to and from Yagaum to the field sites was by a utility vehicle dedicated to the project. All field work involving participants in the study was conducted in the participants’ own villages.

8.2. Data collection and processing

Demographic, clinical and basic laboratory data was recorded on data-sheets by members of the research team at each of the study sites. This included signed confirmation of verbal consent by one of the investigators in the Madang study. Body weight was measured in clothed subjects, without shoes, using bathroom scales (Propert, NSW). Axillary temperatures were measured using a digital clinical thermometer (Terumo Corporation, Japan). Data was entered into Microsoft Access and Excel 97 spreadsheets and the accuracy of data entry was manually cross-checked by comparing printouts of the spreadsheets with the original paper datasheets.

8.3. Overnight fasting protocol

To control for the confounding effect of dietary nitrate ingestion on NOx levels, subjects from Genyem and Madang, and controls from Jayapura and Darwin, were fasted for a minimum of 12 hours overnight following a low nitrate meal of chicken and rice that was provided by the research team. This protocol had been previously shown to account for the confounding effect of dietary NOx [308]. Liberal quantities of nitrate-free water were permitted during this period but smoking and chewing of betel nut were prohibited as their effect on NO production was unknown. In
Genyem, chicken pieces were sourced locally and rice was boiled in town water in a
direct arrangement with the proprietor of a local restaurant. In Madang, frozen
chicken pieces were purchased directly from the Madang Rural Products factory
(which conveniently adjoined the PNGIMR facility in Yagaum) and cooked in
boiling water by the study participants in their own villages. In Darwin, fresh
chicken breast fillets originating from Ingham’s Enterprises were purchased from
Coles supermarket in Casuarina and cooked by a variety of methods, including
baking, steaming and frying. Chicken from the three study sites was confirmed to be
preservative and additive-free through discussion directly with the suppliers and was
cooked then consumed without additives or condiments. Rice purchased from
supermarkets in Madang and Darwin was cooked in boiling water. Water used for
cooking and drinking from the 3 study sites was found to be NOx free after assay
using the method outlined in Chapter 9.2.

Overnight supervision of the Genyem and Madang subjects was performed by one of
the investigators who slept on site at the Genyem health centre or in the Madang
villages participating in the study. Darwin subjects were provided with written take-
home advice sheets informing them of the restrictions and reasons for the fasting
protocol. All subjects were questioned as to whether they had adhered to the fasting
protocol prior to specimen collection the next morning, with the intention of offering
re-enrollment at a later date to non-adherent participants. Blood and urine specimens
were collected as described below after at least 12 hours of fasting.

8.4. Specimen collection and processing

8.4.1. Blood

At the time of initial screening, blood was obtained by fingerprick from all Genyem
and Madang subjects using sterile lancets (Lance, UK), directly applied to glass
microscopy slides and used to prepare a thick and thin blood films. Concurrently,
three blood spots of approximately 50 µL each were directly applied to BFC 180
filter paper (Whatman, US). Glass slides were air-dried in racks and filter papers
were air-dried in a hanging box that ensured separation of one specimen from
another. After drying, filter papers were individually sealed in zip lock plastic bags
and stored at room temperature for up to 4 months before transfer to Australia and long-term storage at 4°C.

Blood was collected by venipuncture into sterile ethylene diamine tetra-acetic acid (EDTA) and heparin-anticoagulated Vacutainer tubes (Becton Dickinson, US) in Genyem and Madang 24 hours after the initial screening and into heparin-anticoagulated tubes only in Darwin. The volumes of blood collected at the different study sites were: Genyem 28 mL; Madang – children ≥ 10 years and adults, 11 mL, children = 9 years, 6 mL; and Darwin adult controls, 12 mL. A second thick and thin film was immediately prepared in all subjects enrolled in Genyem and Madang from a drop of whole blood extracted from the blood collection system tubing prior to exposure to anticoagulant. An aliquot of plasma was separated by centrifugation from EDTA-anticoagulated blood and stored at -20°C within 20 minutes of collection in Genyem.

Hemoglobin levels in Genyem and Madang subjects were measured in EDTA-anticoagulated blood using the Coulter counter in the NAMRU-2 laboratory or a bench top B-hemoglobin analyser (HemoCue, Sweden) calibrated daily against the standard in the Yagaum laboratory. Total white cell counts (WCCs) in EDTA-anticoagulated blood from Genyem were measured using the Coulter counter in Jayapura. WCCs in Madang were measured manually in a Neubauer counting chamber after 1:16 dilution of 60µL whole blood in a solution containing 2% glacial acetic acid and a small amount of crystal violet (which lyses erythrocytes without affecting the architecture of leucocytes; [309]). All remaining plasma was separated by centrifugation within hours of collection and stored in aliquots at -70°C. PBMC pellets were stored at -70°C after separation by density gradient centrifugation (Chapter 9.1). The remaining blood components (erythrocytes, granulocytes and platelets) were discarded.

8.4.2. Urine

Urine from the first morning void of all subjects and controls was discarded but the second void urine collected into sterile plastic specimen containers. Dipstick analysis of urine was performed using multiple reagent strips (Genyem and Madang:
Bayer Diagnostics, Australia; Darwin: Roche Diagnostics, Germany). All specimens with urine reagent strip results indicative of possible urinary tract infection (UTI; i.e., positive for leucocytes, nitrites, protein or blood) were examined in a Kova counting grid (Hycor Biomedical, US). The presence on light microscopy of bacteria (other than vaginal flora) in association with pyuria was used as a criterion to exclude that urine from NOx analysis because of potential for under-estimation of NO production due to NO$_3$/NO$_2$ reduction to NH$_3$. Urine specimens from both field sites were frozen at -20°C in two aliquots soon after collection, one of which was neat and the other after adding isopropanol to prevent bacterial overgrowth at a final isopropanol dilution of 1:5.

### 8.5. Transport of specimens

All urine, plasma and PBMC samples from the field were transferred at approximately -150°C in a liquid nitrogen dry shipper (Taylor-Wharton, US) to Darwin and then stored at -70°C until further analysis. Blood spots on filter paper were transported in their sealed bags at ambient temperature and stored at 4°C after returning to Darwin.

### 8.6. Thick and thin field microscopy for malaria

Thick and thin blood smears from all screened and enrolled subjects were stained with 4% Giemsa solution. Two thick and thin blood smears from consecutive days were taken from most enrolled Genyem and Madang subjects to take account of the periodic fluctuation of *P. falciparum* density in particular [20,22]. Smears were defined as negative if no parasites were seen on examination of 100 high powered (×1000 magnification) fields. Parasite counts were initially expressed as a ratio of trophozoites per 200 leucocytes seen on the thick film.

#### 8.6.1. Examination of smears

Subjects in Genyem were enrolled after screening smears were examined at the health centre by a relatively inexperienced local microscopist, whose results were later discarded in favor of those read by expert microscopists [38]. All slides from
enrolled subjects were later re-examined blindly by the Indonesian National Centre for Disease Control’s head microscopist (Sri Suprianto) who had 24 years’ experience and independently by a second microscopist from the Royal Darwin Hospital with approximately 20 years’ experience. It was not possible for both microscopists to review discordant slides for logistical reasons, therefore parasitemia was defined on the basis of the combined readings of the two microscopists and then again separately according to the readings of the more experienced microscopist. Statistical analyses were then performed in parallel (Chapter 11.3). Parasitemia was calculated by converting the parasite count (per 200 leucocytes on the thick film) to parasite density (trophozoites/µL of whole blood) with reference to the total WCC as measured on the Coulter counter.

Madang screening smears were initially examined by a field microscopist with > 15 years experience (Moses Lagog) to enable selective enrollment of parasitemic and aaparasitemic subjects. The same microscopist re-examined all smears from enrolled subjects without reference to the initial result. A random 10% of all smears (generated in Microsoft Access), as well as all smears with a parasite species present at a count of ≤ 5 per 200 leucocytes, were re-examined blindly by a second experienced microscopist (Kerry Lorry) with > 15 years experience. Concordant slides were defined as those in which the two microscopists independently agreed on the absence of parasites or species of parasite(s) present, and in which the highest parasite count was less than double the lowest (unless both parasite counts were ≤ 5). Discordant slides were re-examined by both microscopists independently and then by both microscopists together if necessary to reach a concordant result. Once concordance was reached, the parasite counts of both microscopists were averaged and then converted to a parasite density (trophozoites/µL of whole blood) with reference to the manually calculated WCC (Chapter 8.6.2).

In all, 324 (59%) of 546 smears from enrolled Madang subjects were cross-checked. Concordance between the two microscopists after the first round of cross-checking was 69%. This compared favorably to a previous report from the same laboratory [22], especially given the bias toward selection of asymptomatic parasitemic subjects (many with multi-species infections) and inclusion of adults with very low
parasitemias. As the rate of concordance was generally high and the parasitemias of the remaining positive slides generally higher, the first microscopist’s readings were accepted for the remainder of the slides without additional cross-checking.

8.6.2. Derivation of white cell counts and enumeration of parasite densities in Madang

Parasite counts from Madang were converted to parasite densities (trophozoites/µL) with reference to an individual’s own manually calculated WCC from whole blood collected on the same (or next) day in most instances. Manual WCCs were not available for the first 2 weeks of the study for logistical reasons (equivalent to 46 parasitemic subjects at enrollment [assigned a notation of T₀] and 1 at first follow-up [notated T₁]; Chapter 12.2) and in 7 parasitemic subjects at enrollment whose first visit was washed out by heavy rain (Chapter 12.3.1.4). Three options were considered for assigning a WCC in these subjects: using a WCC from the same subject calculated at a subsequent date; using an age-adjusted WCC derived from the study population at the same visit; or using a non-age-adjusted arbitrary WCC derived from a different population, as commonly used in malaria publications [22].

WCCs from T₀ were significantly higher than WCCs from T₁ in the same individuals (median difference 800×10⁶/L; P=0.006; paired Wilcoxon test), which may have been an effect of drug treatment. The median absolute difference in WCCs from T₀ compared to T₂ in 50 subjects not excluded at either time-point (Chapter 12.2), and whose first WCC was measured manually, was 80×10⁶/L (P=0.78; paired Wilcoxon test). The magnitude of this difference was only 1.2% of the corresponding median WCC from T₀ of 6860×10⁶/L. As the difference in paired WCCs between T₀ and T₂ was negligible and not statistically significant, and the two counts were strongly correlated (rho [?] =0.71; Chapter 10), WCCs from T₂ were substituted for T₀ WCCs in the 29 subjects for whom a measured WCC was available at T₂.

Age-adjusted WCCs for T₀ and T₁ were derived from two separate regression models that included subjects meeting the inclusion criteria for the study whose WCC had been measured manually. Age, grouped 1-4, 5-9, 10-14, 15-19 and 20+ years, was a significant predictor of the natural logarithm of WCC at T₀ (n=116; r²=0.26;
P<0.001) and at T1 (n=155; r²=0.17; P<0.001). Parasitemia with *P. falciparum* in particular or malaria parasites in general was not significantly related to WCC in either of these models. The age-adjusted GM WCCs for each age group at each time-point are given in Table 8.1. As the WCCs differed significantly between age groups and the confidence intervals were relatively narrow (Table 8.1), these age-adjusted WCCs were substituted for the remaining 24 subjects from T0 and 1 subject from T1. This was considered a better alternative than using a non-adjusted WCC derived from a different population.

**Table 8.1. Predicted age-adjusted leucocyte counts in PNG subjects**

<table>
<thead>
<tr>
<th>Time-point</th>
<th>Age 1-4 years¹</th>
<th>5-9 yr</th>
<th>10-14 yr</th>
<th>15-19 yr</th>
<th>20+ yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>8595 (7209-10248)</td>
<td>7584 (6714-8567)</td>
<td>8505 (7530-9607)</td>
<td>5250 (4508-6113)</td>
<td>6045 (5476-6673)</td>
</tr>
<tr>
<td>T1</td>
<td>8988 (7687-10508)</td>
<td>7339 (6592-8172)</td>
<td>8216 (7331-9207)</td>
<td>5740 (4886-6743)</td>
<td>6248 (5723-6821)</td>
</tr>
</tbody>
</table>

¹Given are geometric means and 95% confidence intervals derived from linear regression models.
Chapter 9. Laboratory protocols

In general, stock chemicals were purchased locally from BDH AnalaR, Ajax Chemicals and Sigma. The source of all other reagents, kits and equipment referred to in this chapter is provided where first mentioned in the text. The composition of solutions is also given at the place of first mention in the text. All dilutions were prepared in sterile deionised ultra-filtered H₂O except where otherwise specified.

9.1. Peripheral blood mononuclear cell separation by density gradient centrifugation

Blood was kept at ambient temperature for between 1-4 hours prior to the commencement of this procedure, which was performed at the NAMRU-2 laboratory in Jayapura, the PNGIMR laboratory in Yagaum, and the MSHR laboratory in Darwin.

The original volumes of whole blood from which PBMCs were separated were: Genyem subjects, 20 mL; Madang subjects ≥ 10 years, 11 mL; Madang subjects < 10 years, 6 mL; Darwin controls, 12 mL. Following centrifugation and aspiration of plasma (Chapter 8.4), remaining blood components were rinsed from the collection tube with phosphate buffered saline (PBS; 9.55g/L PBS powder, pH 7.4; Gibco BRL, US) and transferred to a clean test tube by pipette. A final dilution approximately equivalent to 1.5 times the original volume of whole blood was prepared with PBS. This suspension was gently layered over a volume of Ficoll-Paque Plus (Amersham Pharmacia Biotech, UK) equivalent to ? the volume of the suspension, in either a 50 mL (individuals ≥ 10 years) or 15 mL (children < 10 years) centrifuge tube. Tubes were centrifuged at 400 g (measured at the blood/Ficoll interface) for 30 minutes at 20°C and allowed to come to rest without braking.

The mononuclear cell layer was then transferred by pipette to a clean 15 mL centrifuge tube and resuspended in PBS to a total volume of 14 mL. This tube was centrifuged at 100 g for 10 minutes at 20°C, following which the supernatant was aspirated and discarded. Cells were resuspended in the same tube in 10 mL of PBS.
and centrifuged a second time at 100 g. After aspiration of the supernatant, cells were resuspended in 2 mL of PBS and divided approximately equally into two 1.5 mL cryovials. These were centrifuged at 100 g for 10 minutes, after which the supernatant was again discarded, leaving cell pellets which were immediately frozen at -70°C.

9.2. Determination of nitrite plus nitrate by the Griess reaction

NOx was measured using *Aspergillus* nitrate reductase coupled with the Griess reaction using a method published during the course of this thesis [310].

9.2.1. Preparation of samples and standards

In most cases, urine NOx was measured in specimens to which isopropanol had been added in the field at 1:5 dilution (Chapter 8.4.2) to minimise the potential effect of bacteria in reducing nitrate to un-measurable compounds. Stored urine aliquots were thawed, vortexed and centrifuged at 13000 g for 5 minutes to remove any cells, cellular debris and other insoluble particles. The supernatant was diluted 1:10 to a volume ≥ 150 µL and mixed by vortexing.

Heparin-anticoagulated plasma was thawed, vortexed and centrifuged as above, and plasma proteins > 10000 NMWL removed by ultrafiltration of plasma through Ultrafree MC regenerated cellulose filters (Millipore, US) at 15000 g for 90 minutes. Yields of ultrafiltrate were improved by increasing the temperature at which centrifugation occurred from 4°C (Genyem samples) to 15°C (PNG samples). Potential evaporation and concentration of specimens at the higher temperature was excluded by weighing 8 samples before and after centrifugation and confirming no evaporative loss. At 4°C, most specimens required dilution at 2:3 to 5:6 to ensure sufficient volume for assay, whereas this was only required in 8 of 479 samples at 15°C. Sodium nitrite and sodium nitrate (both from Sigma, US) standards were prepared by diluting 1 mM stock solutions to 160 µM for the first standard and then by serial double dilution down to 2.5 µM.
9.2.2. Assay procedure

Initially, 50 µL of samples and standards were added in duplicate to each well of a 96 well cell culture microtitre plate (Nunc, Denmark). To each well was added 7 µL of 1M Tris (pH 7.5), 10 µL of 0.02 mM nicotinamide adenine dinucleotide hydride (NADPH; Sigma, US) and 23 µL of a solution comprising 20 µL of 5mM glucose-6-phosphate (Boehringer Mannheim [BM], Germany) plus 3 µL of 10 U/mL glucose-6-phosphate dehydrogenase (BM, Germany). An aliquot of 10 U/mL Aspergillus nitrate reductase (BM, Germany) stored at -70°C was thawed, diluted 1:10 and then 10 µL added to each well without delay and mixed by pipetting. Plates were incubated in the dark at room temperature for 30 minutes. Following incubation, 75 µL of each sample/standard was added to 75 µL of Griess reagent (solution containing 2.3% concentrated [85%] orthophosphoric acid, 1% sulfanilamide by weight [Sigma] and 0.1% naphthylehylenediamine by weight [Sigma]) in a new microtitre plate and mixed by pipetting. The reaction was developed for 10 minutes and then absorbances were read at 540 nm.

9.2.3. Interpretation and correction of results

The results were accepted if ≥ 90% of nitrate standard was converted to nitrite, and if the relationship between concentration of nitrate standard and absorbance was linear between 10 µM and 160 µM (i.e., \( r^2 > 0.9 \)). Samples with results outside this range were repeated in another assay at a different dilution. Samples with > 10% discordance between duplicates were also repeated in a new assay. Final results were obtained by comparing the absorbance value of samples to the nitrate standard curve and multiplying by the dilution factor. In the case of plasma samples, a correction factor was subtracted from the final result to account for the small amounts of NOx present in the filter units. The correction factor was equal to the average NOx in ≥ 2 ultrafiltrates of 200 µL dH₂O centrifuged through cellulose filters of the same lot number as those used in the assay. These results were small and ranged between 1.63-3.51 µmol/L for different lots. Twelve heparin-anticoagulated tubes from the same lots used for blood collection were found not to be contaminated with NOx (by testing water samples taken from the tubes), therefore no additional correction for plasma samples was applied.
Creatinine (Cr) was measured in urine with an automated dry chemical analyser
(Johnson and Johnson, US) and urine NOx values were routinely expressed as
NOx/Cr ratios to correct for differences in urinary concentration between subjects.

9.2.4. Effect of isopropanol on urine NOx/creatinine ratios

The effect of isopropanol on calculation of the urine NOx/Cr ratio was examined by
comparing results from neat urine to isopropanol-containing urine in 8 subjects from
Genyem and 20 subjects from Madang. None of these subjects had evidence of UTI
on urinalysis or microscopy. NOx, Cr and the NOx/Cr ratio were determined as
above and agreement between the results of paired samples assessed by measuring
variation from the line of perfect agreement using the method of Lin [311]. In both
instances, precision within paired results and accuracy in relation to the line of
perfect agreement was excellent. The concordance correlation coefficients (?c) were
very high and the confidence intervals narrow (Genyem subjects: ?c=0.914; 95% CI
0.780-1.048; Madang subjects: ?c=0.973; 95% CI 0.95-0.996; where ?c=1 represents
perfect agreement). It was therefore concluded that isopropanol had no significant
effect on the calculation of urine NOx/Cr ratios and that results from neat or
isopropanol-containing urine could be used interchangeably. Neat urines were used
in 16 of 474 Madang subjects without urinary tract infections whose isopropanol-
containing urine had been lost in transit from PNG.

9.3. Measurement of NOS activity by the arginine to citrulline conversion assay

The activity of NOS was measured in PBMC pellet lysates by measuring the amount
of [14C]arginine that was converted to citrulline per mg of total protein per hour [94].
Prior to the assay, a cation exchange resin (Supelco, US) was prepared by adding
NaOH to a strongly acidic exchange resin and then checked for retention of
[14C]arginine. PBMC pellets were retrieved from storage at -70°C, lysed,
centrifuged and then assayed for protein. A reaction mix containing [14C]arginine
(NEN Life Sciences, US) and the essential co-factors Ca^{2+} (from CaCl_2), flavin
adenine dinucleotide (FAD; Sigma, US), tetrahydrobiopterin (THB_d; Sigma, US) and
NADPH (Sigma, US) was then added to the cell lysates and incubated for 1 hour at
37°C. Following incubation, reactions were terminated by inactivating NOS with a buffer containing EDTA (Sigma; US) and then applied to columns of the exchange resin, to which positively charged arginine tightly binds [312]. Citrulline, which carries a neutral charge at pH 5.5, passes through the resin and then NOS activity is quantitated by measuring the radioactivity in the eluate. Negative and positive controls (untreated and LPS/IFN-γ-stimulated cells from the murine macrophage cell line J774) were included in each assay and results were expressed as the number of pmol of $[^{14}\text{C}]$citrulline that were produced per mg of protein in the lysate.

### 9.4. Prostaglandin-E$_2$ ELISA

Measurement of PG-E$_2$ metabolites in urine was attempted using a commercial enzyme linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (Cayman Chemical, USA). A number of problems were encountered with this kit, including: unacceptable batch to batch variability between kits; lack of reproducibility of results from the same sample from one assay to another; lack of agreement between results run on the same samples at different dilutions and the likely presence in concentrated urine of inhibitors to the reaction. In addition, the manufacturers instructions were changed during the period of time in which troubleshooting of the assay was occurring, and standards needed to be replaced by the company on a number of occasions because of degradation. Finally, it was decided that the results from this assay were not reliable enough to be meaningful and after a lot of troubleshooting, the assay was abandoned.

### 9.5. Whole genome amplification by PCR

#### 9.5.1. Purification of DNA

Two 3 mm diameter disks (approximately 3 µL of whole blood per disk) were punched from filter paper blood spots (Chapter 8.4) onto a clean tissue and transferred with a 19 g needle to a 96 well cell culture microtitre plate. The hole punch was cleaned between donors by punching 5-6 blank disks from a piece of uncontaminated blotting paper. Two blank disks were included in the microtitre plate after every 5th sample as negative controls. The disks were washed by adding
100 µL of DNA purification solution (Gentra, US) to each well and incubating overnight at room temperature. The solution was removed the next morning and discarded after first mixing by pipetting up and down 3 to 4 times. Three more washes were performed using 100 µL fresh purification solution each time with 15 minutes between each wash. The last 2 washes were done using 200 µL of 100% ethanol for 1 minute each. Disks were air dried for 3 hours then stored at either 4°C (short term) or at -70°C for longer term storage.

9.5.2. Whole genome amplification PCR

Two disks for each subject and negative control were transferred to PCR tubes and soaked in 50 µL of a reaction buffer (1× whole genome amplification [WGA] buffer) containing 67 mM TrisHCl, 2.5 mM MgCl, 16 mM (NH₄)₂SO₄ and 0.01% Tween-20 for 15 minutes prior to adding the master mix. PCR amplification was performed in 50 µL reactions containing 0.1 mM dNTPs; 6 ng random decamers (Geneworks Gigaprim Kit, Vic); 1× WGA buffer and 5 U HotStarTaq polymerase (Qiagen, Vic). Samples and controls were initially held at 92°C for 15 minutes to activate the HotStarTaq and then cycled 50 times at 92°C for 1 minute, 37°C for 2 minutes and 55°C for 4 minutes (after a 10 second ramp to 55°C).

9.5.3. Confirmatory PCR

A second PCR was then performed using 2.5 µL of product from the WGA reaction added to 50 µL of reaction mix containing 0.2 mM dNTPs; 1X PCR buffer (Qiagen, Vic); 1 U HotStarTaq and 1 µM each of primers for a segment of the human histidyl-tRNA synthetase gene [313]; (5’-3’): ACT GTC GAC CTT CAG GGA GAG CGC GTG CG and ACT GTC GAC TCA TCA GGA CCC AG C TGT GC. Samples were initially held at 95°C for 15 minutes then cycled 34 times at 55°C for 30 sec, 72°C for 1 minutes, 95°C for 30 sec, followed by one incubation each at 55°C for 30 sec and 72°C for 5 minutes. The reactions were analysed by 1.5% agarose gel electrophoresis in 1× TAE buffer (4.84 mg/mL Tris, 0.11% glacial acetic acid, 1 mM EDTA) containing 0.5 µg ethidium bromide per mL. Visualisation of a 400-bp band confirmed the presence of genomic DNA.
9.6. Genotyping of Interleukin-12B 3’UTR and promoter polymorphisms

Two separate polymorphisms in the \( IL12B \) gene were detected by PCR. The 3’ UTR single nucleotide polymorphism represents an A to C change in exon 8 (the 3’ UTR) of the \( IL12B \) gene at position 16974 of the \( IL12B \) sequence in Genbank entry AY008847 [314]. The primers used to amplify this region were (5’-3’): TTT GGA GGA AAA GTG GAA GA and AAC ATT CCA TAC ATC CTG GC. PCR products were digested with \( Taq \) I endonuclease and resolved on polyacrylamide gels. Digestion products indicated the presence of \( IL12B \)-3’UTR allele-2; undigested products represented allele-1.

The promoter polymorphism (\( IL12B \)-pro) is a complex insertion-deletion polymorphism 3 kb upstream of the transcriptional start site that results in a fragment (allele-2) that is 4 base pairs shorter on PCR than allele-1. The primers used to amplify this region were (5’-3’): TCA GAC ACA TTA ACC TTG CA and TAA TGT GGT CAT TGG CAG GT. The amplified sequence spans bases 10188-10468 of the chromosome 5 clone CTB-9P16 sequence in Genbank entry AC011418 (direct submission: DOE Joint Genome Institute and Stanford Human Genome Center, US).

9.6.1. PCR amplification

The template for both reactions was DNA product from the WGA PCR (Chapter 9.5.2) diluted 1:7 in TE4 buffer (10mM Tris pH 8.0; 0.001 mM EDTA) and then 1:2 in H\(_2\)O. PCR amplification was performed in a 5 µL reaction mix containing 2.5 µL diluted WGA product; 1× PCR buffer (Gibco BRL, Vic); 0.2 mM dNTPs; 2 mM additional MgCl\(_2\); primers at 2.9 nM and 0.6 nM concentration for the \( IL12B \)-3’UTR and \( IL12B \)-pro PCR respectively; 0.1 U \( Taq \) polymerase (Gibco BRL, Vic) and incorporating [\( a^{-32}P \)]dATP (Geneworks, Vic). Samples were incubated in 192 well microtitre plates at 95°C for 2 minutes, 60°C for 25 sec, 72°C for 2 minutes and then cycled 35 times at 95°C for 20 sec, 55°C for 15 sec and 72°C for 30 sec (\( IL12B \)-pro) or 40 sec (\( IL12B \)-3’UTR), followed by a final extension at 72°C for 1 minute.
9.6.2. Restriction enzyme digestion

PCR amplified product from the *IL12B*-3'UTR PCR was digested by adding to each sample an equal volume of reaction mix containing 2× digestion buffer, 0.4% bovine serum albumin (BSA), approximately 0.6 U *Taq* I endonuclease (all from Promega, US) and H₂O. The mixture was incubated at 65°C for 2 hours.

9.6.3. Detection of polymorphisms

Wells were made up to 16 µL (*IL12B*-3'UTR) or 15 µL (*IL12B*-pro) with loading buffer and samples denatured at 95°C for 3 minutes. Radio-labelled PCR product was resolved by polyacrylamide gel electrophoresis using a 5% running gel containing 39% urea. Resolved products were analysed visually after exposing X-ray film overnight at -70°C.

9.7. Genotyping of the *NOS2* G-954C polymorphism

The G-954C polymorphism represents a single nucleotide change from G to C at position 2862 in the NOS2 promoter sequence defined in Genbank entry Z49251 [315], which is 954-bp upstream of the transcription start site defined in Genbank entry D29675 [316]. Presence of the polymorphism results in resistance to *Bsa* I digestion at this site.

9.7.1. First round PCR Amplification

Product from the WGA PCR (Chapter 9.5.2) was diluted 1:10 in H₂O for use as the template in amplifying a 680-bp fragment of the NOS2 promoter region spanning bases 2676-3355 of the NOS2 promoter region [315]. PCR amplification was performed in a 50 µL reaction mix containing 5 µL of diluted WGA PCR; 1× PCR buffer (Qiagen, Vic); 4 mM total MgCl₂; 0.2 mM dNTPs; 200 nM of each primer ([5'-3']: CTC AAC TTC TCC CTA ATG TAG TTG and TGC CCA GGT TCC AGA AGG CCA G) and 1.25 U HotStarTaq polymerase. Samples were initially incubated at 92°C for 3 minutes and then cycled at 92°C for 30 sec, 60°C for 1 minute and 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes.
The reactions were analysed by 3% agarose gel electrophoresis in 1× TAE buffer containing 0.5 μg/mL ethidium bromide (Boehringer Mannheim, Germany).

9.7.2. Second round PCR amplification

Weakly amplified samples were re-amplified using 5 μL of product from the first round PCR as the template with internal primers specific to a 573-bp fragment spanning residues 2727-3299 of the NOS2 promoter region ([5’-3’]: CAT ATG TAT GGG AAT ACT GTA TTT CAG and TCT GAA CTA GTC ACT TGA GG; [315]). The procedure for the second round PCR was otherwise identical to that for the first round PCR.

9.7.3. Restriction enzyme digestion

Product from one of the previous two PCR reactions was digested in a 50 μL reaction mix containing 20 μL of amplified DNA; 1× digestion buffer; 1 U Bsa I (both from New England Biolabs, US) and H2O at 50º for 2 hours. The mixture was then incubated at 65°C for 20 minutes to inactivate the enzyme and the product resolved by 3% agarose gel electrophoresis as above.

9.8. NOS2 promoter pentanucleotide microsatellite polymorphism assay

The number of CCTTT repeats in the NOS2 promoter ~2.5 kb 5’ to the transcription start site was determined in WGA DNA product (Chapter 9.5.2) as previously described [317]. PCR was used to amplify an approximately 197-bp fragment spanning residues 1076-1272 in the NOS2 promoter sequence defined in Genbank entry Z49251 [315]. The primers used were (5’-3’): ACC CCT GGA AGC CTA CAA CTG CAT and GCC ACT GCA CCC TAG CCT GTC TCA; and the reaction mix included a trace amount of [γ-32P]ATP-labelled ACC CCT GGA AGC CTA CAA CTG CAT primer. After cycling, PCR product was resolved on an acrylamide/urea gel by electrophoresis, exposed to X-ray film and gels read independently by 2 observers.
9.9. ELISA measuring IgG and IgM antibodies to *Plasmodium falciparum* glycosylphosphatidylinositols

Anti-GPI IgG and IgM antibodies were measured by ELISA. The GPIs used in this study were purified by high performance liquid chromatography (HPLC) of the *P. falciparum* GPI-enriched fraction obtained by solvent extraction procedures as described previously [140]. The purity of the HPLC-purified GPIs was confirmed by carbohydrate compositional analysis and thin-layer chromatography of similarly purified fatty acid-labelled GPIs. The specificity of the seroreactivity of the HPLC-purified GPIs has previously been established [140]. The GPIs were coated at 1 ng and 2 ng/well, for IgG and IgM assays respectively; these coating concentrations give saturated levels of seroreactivity (D Channe Gowda, personal communication). Tween-20 was included in the blocking buffer at a concentration that reduced nonspecific binding (which was evident in a number of subjects in preliminary experiments) without affecting the overall seroreactivity of samples or adherence of [³H]glucosamine-labelled GPIs to the ELISA plate (D Channe Gowda, personal communication).

9.9.1. Procedure

A stock solution of HPLC-purified GPIs was diluted with methanol and coated at 25°C overnight onto half of a 96 well Maxisorb polystyrene microtiter plate (Nunc, Denmark). Plates were either used immediately after coating or stored in an air-tight bag at 4°C for use up to 4 weeks later. Plates were washed once with 5% non-fat dairy milk in phosphate-buffered saline, pH 7.2 (PBS-NFDM) containing 0.05% Tween-20 (used for all subsequent washes and dilutions) and then blocked with 250 µL PBS-NFDM containing 0.5% Tween-20 at 25°C for 2 hours. 50 µL of samples and controls (below) were added in duplicates at 1:100 dilution for IgG and at 1:25 for IgM to GPI-coated and uncoated wells on the same plate and then incubated for 1 hour at 25°C. Plates were washed 5 times and then incubated with 50 µL horseradish peroxidase-conjugated goat anti-human IgG (H & L chains; Kirkegaard and Perry Laboratories [KPL], US) or sheep anti-human IgM μ chain (Chemicon, Vic) at 1:2000 dilution for 1 hour at 25°C. Following 5 more washes (the last 2 in PBS, pH 7.2, containing 0.05% Tween-20), optical densities were read at a wavelength of 405.
nm after 15 minutes incubation with 50 µL ABTS substrate (2,2'-azino-di [3-ethylbenzthiazoline-6-sulfonate]; KPL, MD). Finally, background optical densities from uncoated wells were subtracted from those of GPI-coated wells to adjust for non-specific binding.

9.9.2. Interpretation and correction of results

The optical densities from 15 non-malaria-exposed Australian adult controls (mean age 28 years [range 20-44]; 33% male) were expressed as percentages relative to malaria-exposed adult positive controls for both IgG and IgM. As the results in Australian controls were normally distributed in both assays, values of 2 standard deviations (SDs) above the means were chosen as representing a cut-off between positive and negative and arbitrarily assigned a value of 1. The optical densities from all subjects, after controlling for non-specific binding, were similarly initially expressed as percentages relative to the positive controls and then as multiples of the cut-off in Australian controls (i.e., values > 1 indicating positive results). Positive and negative controls were run on each plate and showed acceptable variability between assays. Longitudinally paired samples from the same individual were assayed concurrently on the same plate.

9.10. Anti-GPI IgG subclass ELISA

9.10.1. Procedure

GPIs were purified from cultured *P. falciparum* as previously described [140], dissolved in methanol and then coated overnight onto a 96-well microtiter plate (Maxisorb by Nunc, N.Y.) at a concentration of 2 ng/well. Plates were washed once with 5% skim-milk in phosphate buffered saline, pH 7.2 containing 0.05% Tween 20 (PBST; used for all subsequent washes and dilutions) and then blocked with 300 µL of 5% skim-milk in PBS containing 0.5% Tween 20 at 25°C for 2 hours. Plasma from subjects and controls was incubated in volumes of 100 µL/well at a dilution of 1:50 for IgG₁ and IgG₃ assays and 1:25 for IgG₂ and IgG₄ assays for 90 minutes at 25°C. One pair of samples was assayed at 1:25 dilution for IgG₁ and 2 pairs of samples were analysed at 1:25 dilution for IgG₃ after initial results at 1:50 dilution.
were shown to be low. Longitudinally paired samples were assayed on the same plate for IgG₁ and IgG₃, whereas only samples from T₀ were assayed for IgG₂ and IgG₄.

Following incubation of samples, plates were washed 5 times and then incubated with 100 µL of horseradish peroxidase-conjugated monoclonal mouse anti-human isotype-specific IgG (The Binding Site, U.K.) at 25°C for 1 hour at the following dilutions: anti-IgG₁ 1:2000; anti-IgG₂ 1:500; anti-IgG₃ 1:500 and anti-IgG₄ 1:1000. Following five more washes (the last two in PBST), optical densities (ODs) were read at a wavelength of 405 nm after 15 minutes incubation at 25°C with 100 µL/well of ABTS and again after a subsequent incubation of 4 hours 45 minutes at 4°C (total 5 hours).

The degree of non-specific binding (NSB) for each of the four IgG subclasses was assessed as previously outlined in Chapter 9.9 by dummy coating half of the ELISA plate with methanol and subjecting both halves of the plate to the same reactions. NSB was measured in this way for 15 paired samples that were assayed for IgG₁ and IgG₃ (i.e., 30 results) and all of the 13 samples from T₀ that were assayed for IgG₂ and IgG₄. After correction for inter-assay variation (Chapter 9.9.2), the median (interquartile range [IQR]) NSB ODs for each of the subclass ELISAs at 15 minutes and 5 hours respectively were: for IgG₁, 0.014 (0.001-0.025) and 0.034 (0.022-0.087); for IgG₂, 0.01 (0.004-0.011) and 0.015 (0.009-0.019); for IgG₃, 0.004 (0-0.013) and 0.016 (0-0.063); for IgG₄, 0.003 (0.001-0.006) and 0.005 (0.002-0.001). As NSB was minimal and much lower than had been experienced with the anti-GPI IgG and IgM ELISAs (Chapter 9.9), it was deemed unnecessary to adjust for NSB in expressing the final results.

9.10.2. Isotype-specific standard curves

Standard curves were prepared to determine working dilutions of isotype-specific secondary antibodies for the anti-GPI antibody ELISAs and to enable quantification and comparison of different IgG subclasses (Figure 9.1 and Figure 9.2). Purified human IgG kappa myeloma protein from each of the four subclasses (The Binding Site, U.K.) was coated at 4°C overnight in carbonate/bicarbonate buffer (15 mM
Na$_2$CO$_3$/35 mM NaHCO$_3$, pH 9.5) in volumes of 100 µL onto a 96-well microtitre plate in doubling dilutions up to 8 µg/mL. Antigen was flicked out, plates washed twice with 5% skim-milk in PBST and then wells were blocked as above, after which they were again washed 2 times and incubated as previously described with the secondary antibodies. Plates were then washed, developed with ABTS and read as above after 15 minutes and again after a total of 5 hours incubation.

![Graph showing optical density vs Immunoglobulin (kappa) concentration](image)

**Figure 9.1. Anti-GPI IgG subclass-specific standard curves after 15 minutes incubation**

Microtitre plates were coated with 100 µL/well of purified human immunoglobulin (kappa) from each subclass and then exposed to isotype-specific secondary antibodies at the following dilutions (stock solution 1 mg/mL): IgG$_1$ 1:2000; IgG$_2$ and IgG$_3$ 1:500; IgG$_4$ 1:1000. Optical densities were read at 405 nm after 15 minutes development at 25°C.
Figure 9.2. Anti-GPI IgG subclass-specific standard curves after 5 hours incubation

Optical densities were read at 405 nm after 5 hours incubation (15 minutes at 25°C followed by 4 hours 45 minutes at 4°C) according to the procedure described for Figure 9.1.

The specificity of each secondary antibody at the working dilutions used in the anti-GPI subclass ELISAs was proven in a checkerboard ELISA that used myeloma protein as the coating antigen at a concentration of 2 µg/mL (Figure 9.3). This concentration was sufficient to produce saturated development of ABTS after 5 hours incubation for each of the four standard curve ELISAs using the appropriately paired secondary antibody. Although there was detectable cross-reactivity between the anti-IgG\(_1\) secondary antibody and IgG\(_2\) and IgG\(_4\) after 5 hours incubation with this antigen concentration, there was minimal cross-reactivity evident for each of the other 10 discordant pairings. Most importantly, cross-reactivity between IgG\(_1\) and IgG\(_3\) was negligible.
Figure 9.3. Specificity testing of subclass-specific secondary antibodies after 15 minutes incubation and again after 5 hours

Each secondary antibody was co-incubated with each IgG subclass antigen at a concentration of 2µg/mL in an ELISA under similar conditions to those used for testing subjects’ plasma. Cross-reactivity was negligible except in the case of anti-IgG\textsubscript{1} antibody which showed weak recognition of IgG\textsubscript{2} and IgG\textsubscript{4}, neither of which were detected in the plasma of subjects (Chapter 15.3.1).

9.10.3. Interpretation and expression of results

A negative (Darwin adult) and positive (PNG adult) control were included on each assay plate for the anti-GPI IgG\textsubscript{1} and IgG\textsubscript{3} assays. As no positive control was available for the IgG\textsubscript{2} or IgG\textsubscript{4} ELISAs, these were performed on the same plate as IgG\textsubscript{1} and IgG\textsubscript{3} controls. The mean ODs of the positive controls were well below the level of saturated development after 15 minutes incubation (ODs of 1.465 and 1.295 for IgG\textsubscript{1} and IgG\textsubscript{3} controls respectively). To control for inter-assay variation, ODs from all samples were multiplied by a correction factor equivalent to the mean OD of all isotype-specific positive controls divided by the OD for the positive control on
each plate. The maximum adjustments required were ±9% for the IgG\textsubscript{1} assay and ±7% for the IgG\textsubscript{3} assay.

ODs and IgG subclass concentrations from the standard curves at 15 minutes and 5 hours were entered into Prism 3.0 software (GraphPad Software, US) and curves of best fit derived using a non-linear regression technique. R-squared values for each of the 8 standard curves exceeded 0.99. Absolute antibody concentrations for the subjects’ samples were computed from ODs by reference to these curves and then multiplication by the dilution factor. All paired samples were read from the same curve. Samples were read from the 5 hours standard curve if the OD at this time was < 1.6 (for both results in the case of paired samples), which was comfortably below the level of saturation (OD > 1.9 for each subclass; Figure 9.2). Samples with an OD > 1.6 at 5 hours were read in preference from the 15 minute curve, all readings from which were taken from the straight section of the curve (an OD = 0.21 for IgG\textsubscript{1} and = 0.39 for IgG\textsubscript{3}). The GM ODs for samples read at 15 minutes and 5 hours respectively were 0.67 and 0.37 for IgG\textsubscript{1}, and 0.77 and 0.37 for IgG\textsubscript{3}. In the case of IgG\textsubscript{1} and IgG\textsubscript{3}, final concentrations less than the lower level of detection were assigned values halfway between this level and zero (one T\textsubscript{0} and 6 follow-up specimens for IgG\textsubscript{1}, and 2 follow-up specimens for IgG\textsubscript{3}).

Reading ODs after 15 minutes and again after 5hr incubation allowed direct determination of antibody concentrations for the majority of samples assayed for IgG\textsubscript{1} and IgG\textsubscript{3} at a single dilution, thus conserving the limited supply of antigen. A single reading at 15 minutes would have led to inestimable results in samples with very bw ODs, whereas a single reading at 5 hrs would have produced unreliable results in samples with saturated ODs. The validity of this approach was suggested by the uniform and reproducible development of standard curves over time and was further assessed by comparing antibody concentrations calculated from the curves at both time-points for the same samples. The level of correlation and agreement (Chapter 9.2.4) between antibody concentrations calculated at both time-points for samples with ODs on the linear section of both OD versus concentration curves was extremely high ($r = 0.99$ and 0.98 for IgG\textsubscript{1} [Figure 9.4] and IgG\textsubscript{3} [Figure 9.5] respectively; P<0.001 for both).
Figure 9.4. Correlation and limits of agreement between absolute IgG₁ antibody concentrations read from standard curves after 15 minutes and 5 hours incubation

Shown are results for all samples with an interpretable OD at 15 minutes (y axis) and an OD at 5 hours < 1.6 (i.e., below the level of saturation; x axis). Correlation between paired readings is shown as a solid line; the line representing perfect agreement is dashed.
Figure 9.5. Correlation and limits of agreement between absolute IgG₃ antibody concentrations read from standard curves after 15 minutes and 5 hours incubation

Shown are results for all samples with an interpretable OD at 15 minutes and an OD at 5 hours < 1.6 (i.e., below the level of saturation). Correlation between paired readings is shown as a solid line; the line representing perfect agreement is dashed.
Chapter 10. Statistical methods

The general statistical principles and tests described here have been used throughout the thesis. Statistical tests particular to only one chapter of the thesis have been described in that chapter. All statistical analyses were performed using Stata computer software versions 6.0 and 7.0 (Stata Corporation, US). Two sided P values <0.05 were considered to indicate statistical significance. P values <0.05 are given to 3 decimal places, P values of =0.05 but =0.20 are given to 2 decimal places, and P values of >0.20 have either been omitted in the case of analyses said to be not significant or alternatively given without further explanation as an indication of their non-significance. The distribution of all continuous data was checked by visual inspection of histograms and kernel density plots and by using the Stata command “sktest” for skewness and kurtosis. In the event that non-parametric data was readily transformable to normality, that data has been transformed and examined using parametric tests. Otherwise, tests appropriate for non-parametric data have been used. Occasionally, non-parametric data that was readily transformable has been examined using non-parametric tests to ensure consistency in sub-group analyses of the same data. In general, the descriptive summary statistics indicate which types of statistical tests were used (below).

10.1. Descriptive and summary statistics, and box and whisker graphs

The central tendency and dispersion of normally distributed continuous data have been described by their mean and SD; logarithmically transformed data by their GM and 95% confidence interval; and non-parametric data by their median and inter-quartile range. Descriptive statistics for proportions are either given as a percentage alone or with 95% binomial confidence intervals where appropriate. In the PNG studies, age was stratified in sub-groups consistent with earlier studies of malaria immunoepidemiology (1-4, 5-9, 10-14, 15-19 and = 20 years) [18]. Stratification of other variables for use in statistical tests particular to only one chapter have been described in that chapter. A number of box and whisker graphs appear throughout the thesis. In all cases: the top and bottom of the box represent the 75th and 25th centiles respectively. The line through the middle of the box in the median. The
whiskers extend no further than the furthest data point or a distance of 1.5 times the IQR from the box, whichever is greatest. Outlying values greater than 1.5 times the IQR from the box are represented as circles.

10.2. Statistical analyses

10.2.1. Continuous data
For normally distributed continuous data, bivariate analyses were performed using Student’s $t$ test after first checking the equality of variances (SDs) using the Stata “sdtest” command. The Mann Whitney U test was used in place of Student’s $t$ test for non-parametric data. One-way analysis of variance (ANOVA) was used for normally distributed categorical variables with more than 2 levels, and the Kruskal Wallis test instead for non-parametric data. Multivariate analyses of normally distributed continuous data were performed using ANOVA and/or linear regression, whereas non-parametric data was stratified and examined using Mann Whitney U tests. Longitudinal data involving paired observations were analysed using paired $t$ tests or the paired Wilcoxon test as appropriate for the distribution of data. Longitudinal data involving more than 2 observations per subject was analysed using ANOVA for repeated measures. The strength of linear association between two continuous variables was assessed by testing Pearson’s correlation coefficient $r$ against zero for normally distributed data or Spearman’s rank correlation coefficient “rho” (denoted as “$\rho$”) against zero for non-parametric data.

10.2.2. Proportions and binary outcome data
Proportions were examined using binomial exact testing (Stata “bitest”) in the case of single variables with only two levels (e.g., gender) and $\chi^2$ or Fisher’s exact testing for multiple categorical variables depending on the number and distribution of observations [318]. The $\chi^2$ test for trend has been used in place of the general $\chi^2$ test for 2×2 testing involving an ordinal categorical variable. Multiple logistic regression with likelihood ratio testing has been used to model the predictive effect of continuous, ordinal and categorical covariates on binary outcome measures.
SECTION Three.
Field studies
Chapter 11. Nitric oxide production in asymptomatic malaria-exposed adult Papuans

11.1. Introduction
This study was conducted in March 1999 within the framework of the malaria research collaboration between the MSHR and Eastern Provinces of Indonesia. The collaboration was established at inter-governmental level in 1996 to facilitate transfer of knowledge and research into significant infectious disease problems of the region. The original aim of the project was to compare indices of NO production longitudinally in 150 healthy adults with and without malaria parasitemia over 3 months. However, because of increasing political instability in Indonesia in 1999, which included northeast Papua province, it was deemed unsafe to continue the study beyond the first month. The project was therefore scaled down to a cross-sectional pilot study comparing indices of NO production in adults with asymptomatic malaria infection to a parasitemic healthy controls, with the aim of testing hypotheses that could subsequently inform longitudinal studies. The data gathered from this study were subsequently used to plan the longitudinal study in children and adults in Madang, PNG (Chapter 12).

The specific hypotheses to be tested in this study were that, in adults:

- Systemic NO production is elevated in subjects resident in an environment where malaria and other potentially NO-inducing infections are prevalent relative to controls from a developed country.
- *P. falciparum* and *P. vivax* infection induce NO production in a “dose-dependent” manner depending on the level of parasitemia.
- The main source of systemic NO production in highly malaria-endemic populations is circulating mononuclear cells.
- As IgE and Th1 cytokines may both potentially induce NO production, levels of IgE and IL-18 (as a representative Th1 cytokine) are positively related to NO production.
These hypotheses were addressed by measuring systemic NOx and circulating mononuclear cell NOS activity in subjects from a region where malaria is highly endemic (rural Papua province), and in controls from the provincial capital (Jayapura) and the capital of the Northern Territory (Darwin).

11.2. Methods

11.2.1. Study site

11.2.1.1. Papua Province and Genyem

Papua is the eastern-most province of the Indonesian archipelago with an area of 421981 sq km, equating to 22% of the nation’s land mass [319]. The province is situated between the Equator and 9.5° of latitude south and forms the western half of the island of New Guinea. Its climate reflects its geographic diversity, being predominantly tropical in the heavily forested lowlands with a rainy season between November and May but temperate in the highlands and mountainous regions, which rise to almost 5000 metres.

The population of 2.1 million in 1998 (39% under the age of 15 years; [320]) includes over 800000 mainly Javanese migrants settled under a government-sponsored trans-migration program that began in 1964. The remainder of the population are indigenous and of similar ancestry to those in the eastern half of the island of New Guinea and other Melanesian peoples of the Pacific. The provincial annualised population growth rate was 2.8% between 1995 and 1998, with an infant mortality rate of 49 per 1000 live births and a life expectancy at birth of 64 years in 1997 [320]. Bahasa Indonesia is the official language in the province and was understood by all participants in the study.

The sub-district of Genyem is situated amongst hills and forest approximately 65 km west of the provincial capital Jayapura and 25 km inland from the north coast. Most indigenous people in the region are subsistence agriculturalists inhabiting small family based villages. Housing in these villages is generally of traditional wooden construction offering little protection from mosquitoes. The region also includes a
number of transmigrant settlements which are larger and more modern in construction than the traditional Papuan villages and located further away from the forest. Ten indigenous villages within 16 km radius of Genyem were included in the study.

11.2.1.2. Malaria in Genyem subdistrict
The most comprehensive malariometric survey conducted in Papua to date was performed in the Genyem sub-district from November to December 1998 by Emiliana Tjitra [321]. Malaria transmission is reported to be perennial in this region but more intense during the rainy season [321]. Spleen size was recorded and fingerprick blood smears taken on a single occasion were examined in 2217 children under 10 years of age. The overall slide positivity rate for asexual parasitemia was 36% (mesoendemic) whereas the spleen rate was 51% (hyperendemic). *P. falciparum* alone was present in 69% of the positive smears; *P. vivax* in 23%; *P. malariae* in 1% and *P. falciparum*/*P. vivax* combined in 6%. Parasite densities were higher in children aged 1-4 years than in those aged 5-9 years and 41% of the children were reported as having a fever. These rates are inclusive of non-indigenous transmigrants and are thought to under-estimate the true rates in indigenous Papuans due to less frequent use of bed nets and closer proximity to the forest in the indigenous population [321].

In a separate study between February and May 1999 that followed the malariometric survey, no cases of severe malaria were observed in 526 subjects with clinical malaria [321]. The severity of clinical malaria was found to be similar in Genyem children and adults and it was not possible to clinically distinguish malaria caused by *P. falciparum* from that caused by *P. vivax*.

11.2.2. Subjects and controls

11.2.2.1. Screening and enrollment of Genyem subjects
All healthy adults aged 16 years and over living within the study area were eligible to be screened for entry into the study. Screening was undertaken in the morning in one
village per day which was both logistically feasible and sufficient to ensure an adequate number of enrollees. After obtaining written consent, volunteers presenting for screening underwent a clinical assessment questionnaire administered by the study team, measurement of axillary body temperature using a digital clinical thermometer, and collection of thick and thin blood smear by fingerprick.

Clinical criteria for enrollment were: no fever history or treatment for malaria within the past week; no clinical evidence of malaria or other infection; no diarrhea; and no current pregnancy. Screened subjects who fulfilled these criteria were provisionally allocated into one of three study groups on the basis of screening microscopy – asymptomatic *P. falciparum*, asymptomatic *P. vivax* and microscopically negative healthy controls. Six enrollees per day were then selected from those eligible with the aim of ensuring balanced numbers of parasitemic and aparasitemic individuals and approximately equal sex and age distributions in each group. Enrolled subjects were transported back to the health centre in the evening where clinical details were confirmed at interview and measurement of axillary temperature was repeated. A third axillary temperature was recorded the following morning.

### 11.2.2.2. Enrollment of Jayapura and Darwin controls

Controls were recruited in Jayapura between July 1999 and July 2000 as part of a related study of NO production in subjects with clinical malaria. Adult volunteers were recruited in urban Jayapura using the same enrollment criteria as in Genyem and were given the same low nitrate dinner followed by a similar fasting regimen as in Genyem. This fast was unsupervised but adherence was confirmed on questioning the following morning. Temperatures were measured and venous blood and urine were collected prior to breakfast, for the same measurements as in Genyem.

Adult volunteers were recruited from amongst staff and students at the MSHR over two weeks in February 2000 following separate ethics approval. Similar clinical criteria were used for enrollment as were used in the Papuan and Madang studies (Chapter 11.2.2.1 and 12.2.3): i.e., no clinical evidence of malaria or other infection within the past week; no treatment for malaria within the past week; no diarrhea; and no current pregnancy. Participants were given the same low nitrate meal as the
Genyem subjects and followed the same fasting protocol (Chapter 8.3). Axillary temperatures were recorded on two occasions approximately 16 hours apart.

11.2.3. Laboratory assays
Blood smears, plasma and urine NOx, and PBMC NOS activity were examined and assayed as described in Chapter 9. Plasma Cr and plasma IgE were measured on an automated analyser by Ian Gardner of Queensland Medical Laboratories. IL-18 was measured in-house using a commercial kit (Medical and Biological Laboratories, Japan).

11.2.4. Statistical methods
Statistical analyses were performed and are presented as generally described in Chapter 10. As it was not possible for the two expert microscopists to review discordant slides (Chapter 8.6.1), two parallel statistical analyses were performed when comparing the effect of malaria parasitemia on NO production which are presented sequentially. In the first, parasitemia was classified according to the combined readings of the two microscopists and the analysis was further sub-divided according to whether either microscopist observed parasitemia or on the basis of agreement between the two microscopists. In the second, parasitemia was classified solely according to the readings of the more experienced microscopist. The purpose of performing the statistical analyses in this way was to ensure that potential errors of inclusion or omission would be minimised, thus avoiding to the maximum extent possible the chance of type I and type II statistical errors in formulating the hypotheses to be tested in Madang.

11.3. Results

11.3.1. Baseline characteristics

11.3.1.1. Genyem subjects
The total number of volunteers screened in the 11 villages was 179, of which 55% were male (P=0.18) and 97% were of coastal Melanesian ethnicity. The age
distribution of these volunteers was bimodal with peaks at approximately 20 and 40 years of age and a median age of 32 years (IQR 22-41). Parasites were seen on microscopic examination of thick and thin films by the screening microscopist in 34% of subjects: 28% had \textit{P. falciparum}, 5% \textit{P. vivax} and 1 subject had \textit{P. malariae}.

The 56 subjects enrolled in the study were significantly younger (median age 25 years; IQR 20-35) than those excluded from enrollment (median age 36 years; IQR 23-42; P=0.002) but there was no significant difference in distribution of sex.

Volunteers who reported symptoms of ill health in the two weeks prior to screening were significantly less likely to be enrolled in the study (P<0.001) and were significantly older (median age 37; IQR 28-42) than healthy subjects (median age 29 years; IQR 19-38 years; P<0.001). There was no significant difference in age between those who were parasitemic or apasitemic on microscopy.

Sixteen of the 56 enrolled subjects met the exclusion criteria subsequent to their initial screening. Three of these excluded subjects had a measured axillary temperature = 37.5°C, 9 gave a history of fever, 12 gave a history of other symptoms consistent with malaria and 4 reported recent ingestion of antimalarial medication.

One other subject had a UTI and was excluded from analyses involving urine results. There were no significant differences in age or sex between the 40 enrolled subjects who were included in the statistical analyses and the 16 who were excluded.

Twenty three of the 40 included subjects were male (P=0.43) and the mean age of subjects (27 years; SD 9) did not differ significantly between males and females.

One subject was from Sulawesi and had been living in Genyem for 2 years, and the remainder were coastal Melanesians. The mean duration of residence in Genyem was 20 years (SD 10), with 25 of the subjects having never lived elsewhere. All subjects were supervised overnight at the Genyem Health Centre and no breaches to the fasting protocol were observed.

Two thick and thin smears taken on consecutive days were available for examination from 50 of the 56 initially enrolled subjects and one smear only was available from 6. On 42 (75%) of 56 occasions the reading of the screening microscopist was concordant with either or both of the expert microscopists and on 35 (63%) of 56 occasions was concordant with the senior microscopist. There was complete
agreement between the expert microscopists on the presence or absence of parasites, and the species present, in 79 (75%) of 106 slides.

There was a similar level of agreement between the expert microscopists on the combined readings from 28 (70%) of the 40 subjects included in the statistical analysis. In these 28 subjects: 12 had only \( P. falciparum \); 1 had only \( P. vivax \), and 15 were aparasitemic. The GM parasitemia in the 12 subjects with \( P. falciparum \) (based on the average of the highest reading of the two microscopists) was 296 trophozoites/\( \mu L \) (95% CI 147-597). In 8 of the remaining 12 subjects, parasites were seen by one microscopist but not the other (2 had \( P. falciparum \), 5 had \( P. vivax \) and 1 had both \( P. falciparum \) and \( P. malariae \)). On 6 of these 8 occasions, a parasite was only seen by the less experienced microscopist, and on the other 2 occasions, only by the senior microscopist (P=0.29). The median parasite count in these 8 instances was 1.5 trophozoites/200 leucocytes (IQR 1-2; approximately equivalent to 60 trophozoites/\( \mu L \)). In the 4 remaining cases, parasites were seen by both microscopists but there was disagreement on the species present. Overall, parasites were seen by either or both microscopists in 25 of the 40 subjects and \( P. falciparum \) was seen in 19 of these instances.

Based solely on the readings of the more experienced microscopist in the 40 subjects included in the statistical analysis: 12 had \( P. falciparum \); 5 had \( P. vivax \); 1 had both \( P. falciparum \) and \( P. vivax \), and 22 were aparasitemic. The GM parasitemia in the 12 subjects with \( P. falciparum \) (highest of two readings if both positive) was 344 trophozoites/\( \mu L \) (95% CI 209-567). The GM parasitemia in the 5 subjects with \( P. vivax \) was 80 trophozoites/\( \mu L \) (95% CI 37-170).

11.3.1.2. Jayapura controls

Of the 43 controls recruited from Jayapura: 14 (33%) were Melanesians from the coastal region; 6 (14%) were Melanesians from the highlands; and the remainder had emigrated from other parts of Indonesia. The median age was 24 years (IQR 18-30) and 23 (53%) were female (P=0.76). Male controls (median age 28 years; IQR 22-32) were significantly older than female controls (median age 18 years; IQR 16-25; P=0.002). There were no significant differences in sex between subjects of different
ethnicities, however, Indonesian immigrants (median age 30 years; IQR 24-33) were significantly older than both coastal Melanesians (median age 19; IQR 16-22; P=0.001) and Melanesian highlanders (median age 17.5; IQR 16-24; P=0.009). No subjects admitted to breaching the fasting protocol. All of these controls were confirmed on microscopy to be free of malaria parasitemia on initial screening in Jayapura and on subsequent cross-checking by the senior expert microscopist who had examined the slides from Genyem. Urine, plasma and PBMC specimens were available from all subjects and there was no evidence of urinary tract infections on dipstick testing or microscopic examination of urine.

11.3.1.3. Darwin controls
Twenty two adult Darwin controls were enrolled (mean age 35 years; SD 8), of which 13 (59%) were females (P=0.52). There was no significant difference in mean age between males and females. No subjects admitted to breaching the fasting protocol and none met any of the exclusion criteria. Urine, plasma and PBMC specimens were available from all subjects and there was no evidence of urinary tract infections on dipstick testing or microscopic examination of urine.

11.3.2. Renal function and fractional excretion of NOx

11.3.2.1. Genyem subjects
Plasma Cr was within the normal range (males: 60-120 µmol/L; females: 50-110 µmol/L) for 22 of the 23 males (one male’s plasma Cr was 140 µmol/L) and all of the 17 females. The GM plasma Cr was significantly higher in males (95 µmol/L; SD 14) than in females (69 µmol/L; SD 8; P<0.001), with sex alone explaining 60% of the variation in plasma Cr. Plasma Cr was not significantly associated with age or weight after adjusting for sex.

The glomerulofiltration rate (GFR; mL/min) of each subject was calculated using the Cockcroft-Gault equation [322]:

\[
GFR = 141 \times \frac{ Scr \times \text{weight in kg}}{72 \times \text{age in years}}
\]
The calculated GFR was normally distributed (mean 89 mL/min; SD 16) and did not differ significantly between males and females. GFR was below the normal range of 80-120 mL/min (standardised against a body surface area [BSA] of 1.73 m²) in 33% of subjects and above the normal range in 1 subject (3%).

The median fractional excretion of NOx (FENOx; calculated by dividing the urinary NOx/Cr ratio by the plasma NOx/Cr ratio) was 30% (IQR 22-45%) and was not significantly different between males and females. FENOx was not significantly related to age, sex, weight or GFR.

11.3.2.2. Jayapura controls

Plasma Cr was within the normal range in all Jayapura controls and was normally distributed in males but not in females. In separate univariate analyses, sex, age and ethnicity significantly predicted plasma Cr, with sex explaining the most variance ($r^2=0.37$). The significant effect of ethnicity was lost after controlling for sex and age, which both remained significantly independently predictive in a multivariate model ($r^2=0.44$; $P<0.001$). In this model, the predicted plasma Cr for a 24 year old male was 81 mmol/L and for a 24 year old female was 70 mmol/L. As the weights of these controls was unavailable, it was not possible to calculate GFR.

FENOx was significantly higher in females (median 37%; IQR 28-48) than in males (median 17%; IQR 13-25; $P<0.001$). FENOx was not significantly related to age nor ethnicity and it was not possible to test the association between FENOx and weight.

11.3.2.3. Darwin controls

The plasma Cr of all Darwin controls was within the normal range and the mean plasma Cr for males (92 µmol/L; SD 12) was significantly higher than for females
(77 µmol/L; SD 8; P=0.001). The magnitude and significance of this difference was similar after adjusting for the non-significant effects of age and weight.

GFR was normally distributed in these controls and was significantly higher in males (110 mL/min; SD 19) than in females (91 mL/min; SD 20; P=0.036). GFR was below the normal range in 2 females (62 mL/min in both) and above the normal range in 2 females and 1 male. There was no significant association between GFR and age.

The median FENOx was 33% (IQR 28-46) and was not significantly different in males and females. FENOx was calculated to be 171% in 1 subject in whom NOx measurements had been repeated in both plasma and urine and were within the range of results for other controls in both instances. Exclusion of this subject from the analysis did not materially alter the results (median 33%; IQR 28-45). There was no significant relationship between FENOx and age, sex, weight or GFR, whether or not the aforementioned outlier was included in the analysis.

11.3.3. Urinary NO metabolite excretion

The median ages of the 39 Genyem subjects (without UTI) and the 43 Jayapura controls were 24 years (IQR 19-33) and 24 years (IQR 18-30) respectively. Urine NOx/Cr was significantly higher in Genyem subjects (median 0.11; IQR 0.07-0.23) than in Jayapura controls (median 0.07; IQR 0.04-0.10; P=0.006). There was no significant relationship between urine NOx/Cr and age, sex or plasma Cr in either group; nor weight in the Genyem subjects. Urine NOx/Cr did not differ significantly according to ethnicity in the Jayapura controls. Urine NOx/Cr was significantly higher in both Genyem subjects (P<0.001) and Jayapura controls (P=0.02) than in Darwin controls (median 0.05; IQR 0.04-0.06).

11.3.4. Plasma NO metabolites

Plasma NOx was higher in Genyem subjects (median 30 µmol/L; IQR 18-44) than in either Jayapura (median 19 µmol/L; IQR 11-31; P=0.008) or Darwin controls (median 11 µmol/L; IQR 7-14; P<0.001). The difference between Jayapura and
Darwin controls was also significant (P<0.001). Plasma NOx and age were not significantly associated in any of the 3 groups, and plasma NOx was not significantly associated with sex in Genyem subjects or Darwin controls. Plasma NOx was significantly higher in male Jayapura controls (median 31 µmol/L; IQR 23-40 µmol/L) than in females (median 12 µmol/L; IQR 10-18 µmol/L; P<0.001). There was no significant association between plasma NOx and FENOx in any of the groups, including the subset of males from Jayapura.

11.3.5. PBMC NOS activity

NOS activity was higher in Genyem subjects (median 4048 pmol/mg; IQR 2781-5048) than in Jayapura controls (median 3343 pmol/mg; IQR 1826-4385) but this difference was not significant (P=0.17). NOS activity was significantly lower in Darwin controls (median 613 pmol/mg; IQR 495-894) than in Genyem subjects and Jayapura controls (P<0.001 for both comparisons). NOS activity was not significantly correlated with plasma NOx or urine NOx/Cr in any of the three study locations, nor was it correlated with age or sex (or ethnicity in Jayapura controls).

11.3.6. Correlation between different measures of nitric oxide production

Urine NOx/Cr and plasma NOx were significantly correlated in both the Genyem subjects (Spearman’s rho [?]=0.72; P<0.001) and Jayapura controls (?=0.4; P=0.008). In both instances, the degree of correlation was higher in males than in females: for Genyem subjects, ?=0.85; P<0.001 in males, and ?=0.57; P=0.02 in females; and in Jayapura controls, ?=0.83; P<0.001 in males, and ?=0.5; P=0.016 in females. Similar results were observed in the Darwin controls (?=0.51; P=0.016), in whom the correlation in males was significant (?=0.83; P=0.005) but in females was not (?=0.46; P=0.117). NOS activity was not significantly correlated with plasma NOx or urine NOx/Cr in Genyem subjects or Jayapura controls.

11.3.7. NO production and parasitemia in Genyem subjects (primary analysis)

The primary analysis was based on the combined microscopy readings of the two expert microscopists (Chapter 11.3.1.1). Both plasma NOx (median 36 µmol/L; IQR
26-48) and urine NOx/Cr (median 0.14; IQR 0.10-0.23) were significantly higher in subjects in whom a parasite was seen by one of the 2 microscopists than in aparasitemic subjects (median plasma NOx 19 µmol/L; IQR 16-36 µmol/L; P=0.041; median urine NOx/Cr 0.07; IQR 0.04-0.08; P=0.011).

Plasma NOx was highest in subjects with *P. falciparum* seen on microscopy by at least one microscopist, intermediate in those with other parasite species and lowest in subjects who were aparasitemic (Figure 11.1). The difference in plasma NOx between *P. falciparum* parasitemic subjects and aparasitemic subjects was significant (P=0.025) but those with other parasites did not differ significantly from either of the other two groups. Urine NOx/Cr was similar in subjects with parasites other than *P. falciparum* on microscopy (median 0.16; IQR 0.09-0.20) and subjects with *P. falciparum* (median 0.14; IQR 0.10-0.24; P=0.227). The difference in urine NOx/Cr between aparasitemic subjects (median 0.07; IQR 0.04-0.08) and those with *P. falciparum* was significant (P=0.040), but the difference between aparasitemic subjects and those with other parasites was not.

![Figure 11.1. Plasma NOx in Genyem subjects (primary analysis)](image)

The width of each box is proportional to the number of subjects.
Considering only those subjects in whom the expert microscopists agreed on the presence and species of parasites: plasma NOx in subjects with *P. falciparum* parasitemia (median 41 µmol/L; IQR 26-53) was significantly higher than in aparasitemic subjects (median 19 µmol/L; IQR 16-36; P=0.028). Similarly, urine NOx/Cr was significantly higher in subjects with *P. falciparum* parasitemia (median 0.13; IQR 0.11-0.32) than in aparasitemic subjects (median 0.07; IQR 0.04-0.08; P=0.010). There was no significant correlation between the density of *P. falciparum* parasitemia and either plasma NOx, urine NOx/Cr or NOS activity in these subjects.

There was no significant difference in NOS activity between Genyem subjects who were aparasitemic compared to those in whom a parasite was seen by either or both of the expert microscopists or in whom *P. falciparum* was the only parasite seen. Similarly, there was no significant difference in NOS activity comparing subjects in whom the two microscopists had agreed on the presence of *P. falciparum* to aparasitemic subjects.

### 11.3.8. NO production and parasitemia in Genyem subjects (secondary analysis)

The secondary analysis was conducted along the same lines as the primary analysis but was based solely on the microscopy readings of the senior expert microscopist (Chapter 11.3.1.1). Both plasma NOx (median 40 µmol/L; IQR 16-36) and urine NOx/Cr (median 0.14; IQR 0.10-0.23) were significantly higher in subjects in parasitemic subjects than in aparasitemic subjects (median plasma NOx 20 µmol/L; IQR 16-36; P=0.020; median urine NOx/Cr 0.07; IQR 0.04-0.20; P=0.049).

Excluding the one subject with mixed infection, plasma NOx was highest in those with *P. falciparum*, intermediate in those with *P. vivax* and lowest in aparasitemic subjects (Figure 11.2). The difference in plasma NOx between *P. falciparum* parasitemic subjects and aparasitemic subjects was significant (P=0.022) but those with *P. vivax* did not differ significantly from either of the other two groups. Urine NOx/Cr was similar in subjects with *P. vivax* parasitemia (median 0.15; IQR 0.14-0.23) and subjects with *P. falciparum* (median 0.13; IQR 0.11-0.29; P=0.916). The difference in urine NOx/Cr between aparasitemic subjects (median 0.07; IQR 0.04-
and those with *P. falciparum* was significant (*P*=0.040) but the difference between aparasitemic subjects and those with *P. vivax* was not. There was no significant correlation between the densities of either *P. falciparum* or *P. vivax* parasitemia and either plasma NOx, urine NOx/Cr or NOS activity.

![Plasma NOx in Genyem subjects (secondary analysis)](image)

**Figure 11.2. Plasma NOx in Genyem subjects (secondary analysis)**

The width of each box is proportional to the number of subjects.

There was no significant difference in NOS activity between aparasitemic subjects and either subjects with any malaria parasite or those with only *P. falciparum* or *P. vivax*.

**11.3.9. NO production and IgE levels**

IgE levels were above the normal range (< 100 kU/L) in 35 of 36 subjects for whom a result was available (median 3350 kU/L; IQR 1425-5800). There was no significant difference in IgE levels between parasitemic and aparasitemic subjects, including those whose parasitemia was due only to *P. falciparum*, and also after restricting the analysis to only those subjects in whom the expert microscopists had
agreed on the presence or absence of malaria species. Plasma IgE was not significantly related to either plasma NOx, urine NOx/Cr or NOS activity.

11.3.10. NO production and Interleukin-18 levels

IL-18 results were available from 26 of the 27 Genyem subjects in whom the expert microscopists had agreed on the presence or absence of malaria parasite species present. IL-18 levels were higher in subjects with *P. falciparum* parasitemia (median 517; IQR 346-1023) than in those without (median 385; IQR 269-681), but the difference was not significant (P=0.15). There was no significant relationship between plasma IL-18 levels and either plasma NOx or urine NOx/Cr, however, IL-18 significantly predicted NOS activity ($r^2=0.31; P=0.003$). Controlling for parasitemia and/or inclusion of another 9 subjects who had IL-18 measured but who met the exclusion criteria did not affect the significance of this relationship (P=0.006).

11.4. Discussion

11.4.1. Interpretation of NOx in relation to renal function and renal handling

It has previously been demonstrated that renal dysfunction may falsely elevate plasma NOx and influence the interpretation of urine NOx excretion, as the majority of NOx is excreted renally [96,100]. It was therefore important to assess renal function in the study cohort prior to analysis of results. Plasma Crs were within the normal range used by the Royal Darwin Hospital for all Jayapura and Darwin controls and for all but 1 Genyem subject whose plasma Cr was 140 $\mu$mol/L. A higher proportion of subjects from Genyem (33%) had GFRs below the normal range as calculated by the Cockcroft-Gault equation for a standardised BSA of 1.73 $m^2$ than did Darwin controls (this measure was not available in Jayapura controls). While this may be indicative of unrecognised renal insufficiency [323], an alternative explanation may be that subjects from Genyem had a BSA less than 1.73 $m^2$ which led to an underestimation of their GFR. Height was not recorded in the present study but mean heights of coastal Melanesians from PNG have previously been reported at 163 cm in males and 153 cm in females [324], which would yield BSAs of 1.63 $m^2$
and 1.49 m$^2$ respectively if applied to the mean weights of males and females in the present study [325]. Recent evidence also suggests that the Cockcroft-Gault equation may lack accuracy in the sub-group of individuals with normal Crs but proven renal impairment [326]. Given these limitations and the fact that only one subject had an elevated Cr (aparasitemic) and another mild proteinuria (parasitemic with \textit{P. falciparum}), it is unlikely that renal dysfunction would have materially affected interpretation of results.

Plasma NOx and urine NOx/Cr were well correlated in all subjects and controls, but at all 3 study sites, these measures were better correlated in males than they were in females. This may be due to greater variability in females than in males of the factors that regulate NOx excretion or volume of distribution. A potential source of variation in females that does not exist in males is the fluctuation in levels of sex hormones that occurs throughout the menstrual cycle. Sex hormones may effect renal tubular function and fractional excretion of some analytes [327,328] but the specific effect on NO metabolites is unknown.

The median FENOx was remarkably similar in Genyem subjects (30%), Darwin controls (33%), and children and adults (both 34 %) from Madang, PNG (Chapter 12.3.2) which would suggest that renal handling of NOx is similar in healthy subjects of different ages and ethnicity. Although the median FENOx in Jayapura controls was similar to these other populations overall (27%), there was a marked and significant difference between males (17%) and females (37%). Although it was not possible to calculate GFR in the Jayapura controls, this difference does not appear to result from renal insufficiency, because plasma Cr was normally distributed and within normal limits in all male controls. The possibility that the Papuan practice of chewing of betel nut with lime and mustard could explain the difference can likely be discounted as there was no significant difference in FENOx between Papuan and non-Papuan controls.

Another sex-related difference, restricted to Jayapura controls, was that plasma NOx was significantly higher in males than in females, despite there being no significant difference between the sexes in urine NOx/Cr or NOS activity. This difference did not appear to result from the lower FENOx in males as there was no correlation
between plasma NOx and FENOx in males. The median plasma NOx in male Jayapura controls was approximately midway between the values seen in aparasitemic and parasitemic Genyem subjects, whereas the plasma NOx of females was very close to that of Darwin controls and lower than that of aparasitemic Genyem subjects. It is possible that the behaviour of the older male controls varied from that of the female controls with respect to exposure to NO-inducing infections or substances. For example, undeclared non-adherence late in the fasting protocol by males could have potentially resulted in an acutely increased plasma NOx with less effect on the urine NOx/Cr. This could have involved ingestion of nitrate rich food or ingestion/inhalation of other substances including NO in cigarette smoke [99] but does not appear due to use of betel nut for the reasons stated in the preceding paragraph.

11.4.2. Effect of parasitemia on systemic NO production and NOS activity

This study was the first to demonstrate increased NO production in asymptomatic adults with malaria parasitemia in parallel with an earlier finding in Tanzanian children [282]. Plasma NOx and urine NOx/Cr were highly correlated in malaria-exposed subjects and significantly increased in subjects with malaria parasitemia in general and *P. falciparum* in particular compared to aparasitemic controls. It is possible that the results observed in subjects with all-cause parasitemia were unduly influenced by the larger number of subjects with *P. falciparum* compared to other parasites. On sub-group analysis, NOx levels in subjects with non-*P. falciparum* parasitemia were closer to those of subjects with *P. falciparum* than subjects without parasites, but the number of subjects were small and the differences statistically insignificant. In contrast to the NOx results, there were no differences evident in NOS activity between aparasitemic subjects and those with *P. falciparum* or all-cause parasitemia.

The nature and magnitude of the differences observed between parasitemic and aparasitemic subjects were not materially altered whether the definition of parasitemia was based on the readings of both microscopists or solely on the readings of the more senior microscopist. Significant results were found in the most restricted subset of subjects whose definition of parasitemia was based on agreement between
the two microscopists with regard to the presence or absence of *P. falciparum*. That these results were mirrored in the less restrictive analyses suggests that any systematic difference between the two microscopists was minor. It seems more likely that the differences in classification that were observed resulted from random variation between the microscopists at the lowest levels of parasitemia, which is supported by the sparse parasite counts observed in the 8 subjects for whom a parasite was seen by one microscopist but not the other.

11.4.3. Stimulation of nitric oxide production in Papuan subjects and controls

The results from this study showed that systemic NO production and PBMC NOS activity was higher in adults living in Papua province than in Darwin. Aparasitemic urban and rural individuals had similar levels of plasma NOx and urine NOx/Cr but levels were significantly higher in those with asymptomatic *P. falciparum* parasitemia. In contrast, NOS activity was similar in Genyem subjects and Jayapura controls and was not increased in those with malaria parasitemia. Furthermore, there was no correlation between NOS activity and either measure of systemic NO production. This evidence suggests that non-malarial stimuli present in Papua but not in Darwin contributed to inducing NO production from PBMCs and other sources at similar levels in urban and rural individuals.

In addition, *P. falciparum* parasitemia appeared to induce NO production from non-PBMC sources that led to an approximate doubling of basal levels. This effect was seen at the level of microscopic detection of parasites (which may suggest a threshold effect if PCR-level infection was common) and no effect was seen with increasing parasitemia. Defining a relationship between the level of parasitemia and NO production is problematic in a small cohort, especially when one or two readings of parasitemia may be a poor reflection of the overall parasite biomass [20]. Levels of the Th1 cytokine IL-18 correlated with NOS activity but not systemic NO production, suggesting that PBMC NOS induction is at least in part co-induced and/or dependent on IL-18 but that this pathway is less important in inducing NO production from other sources. PBMC NOS expression in this setting is most likely mediated by IFN-γ in response to co-stimulation by IL-12 and IL-18, which has been
demonstrated in human monocytes [329] as well as in T cells and NK cells [330,331].

Although adults with heavy malaria exposure may commonly be parasitemic at submicroscopic levels [21], this possibility was very unlikely in Jayapura controls as parasitemia was absent on microscopy in all 43 subjects screened. A number of intestinal parasitic infections have been shown to be endemic amongst individuals living in Papua province (below) that are rarely seen in adults living in Darwin (Wayne Pederick, Darwin manager of Queensland Medical Laboratories, personal communication). These include the helminths *Trichuris trichiura*, *Ascaris lumbricoides*, *Necator americanus* and *Strongyloides* sp., and protozoal infections such as *Entamoeba histolytica*, *Entamoeba coli* and *Giardia lamblia* [332-335]. In the absence of an extremely high incidence of allergic diseases, it is very likely that helminth infections were the major cause of the very high levels of IgE measured in the Genyem subjects [336]. IgE levels were not measured in the Jayapura controls but it is plausible that their rates of intestinal parasitosis are likely to be more similar to the Genyem subjects than the Darwin controls.

IgE can induce NO production by activating monocyte CD23 receptors [89] and a recent study suggested that IgE levels were positively correlated with plasma NOx in Thai adults with cerebral malaria and in controls with uncomplicated malaria. However, the effect of diet and renal dysfunction was not considered in that study [278]. IgE was not associated with any measure of NO production in the present study but the possibility that IgE did induce NOS2 cannot be entirely discounted. It is conceivable that induction of NO production by IgE could become saturated at a threshold IgE level that was exceeded in most subjects or that IgE’s contribution to overall NO production was too low to be detected statistically. It has also been suggested that IgE alone may not be sufficient to activate CD23 and that the presence of IgE complexes may be more important [337].

Although the aforementioned helminths typically induce Th2 immune responses [338,339], a recent study that examined induction of cytokine production by *T. trichiura* and *Ascaris* antigens in the whole blood of children aged 4 to 15 years from Cameroon found “a varied response that does not easily fit the highly polarised
Th1/Th2 paradigm” [340]. Production of the potent NO inducers TNF-α and IFN-γ was common in that study, particularly in the older subjects, which was interpreted as “suggesting a switch to a more chronic infection phenotype”. The parasite determinants and mechanisms underlying the immunomodulatory activities of *Ascaris* are only now beginning to be understood [341]. *E. histolytica* has also been shown to stimulate TNF-α release leading to NO production by IFN-γ-primed murine bone marrow macrophages *in vitro*, which is thought to be part of the host defence against amoebiasis [342].

The possibility that intestinal parasitosis may explain the elevated NO production of apasitemic rural and urban Papuan controls is indirectly supported by evidence from clinical studies. Murray and family reported that Anjouan children heavily parasitised with *A. lumbricoides* experienced “unusual freedom from malaria”, which was revealed in a placebo-controlled trial by the development of clinical malaria attacks in 54% vs 0% of children treated with an ascaricide [343]. Recently it was shown that infection with *A. lumbricoides* was associated with a near-significant (42% reduction; P=0.06) protective effect against cerebral malaria in Thai adults in a dose-dependent manner [337]. More significant dose-dependent protective effects were shown in subjects co-infected with *A. lumbricoides* and hookworm, *T. trichiura* and *Strongyloides stercoralis* [279,337] and the same authors showed that hookworm infection was associated with lower body temperatures during mild malaria [344]. Although the suppressive effect of *Ascaris* was attributed to malnutrition in the Anjouan study [343], the protective effect of helminths against cerebral malaria in Thailand was maintained after correcting for the independently significant protective effect of malnutrition [345] as well as other socio-economic and environmental risk factors [346]. In another study by the same group it was reported that plasma NOx (excluding cases with obvious renal failure and controlling for Cr) was higher in helminth-infected subjects with uncomplicated malaria and high parasite biomass than in non-infected subjects [279]. The relationships between NOx and severity of malaria in these studies was complex and affected by interactions with splenomegaly [277] and levels of soluble CD23 receptor [279].

It has been observed that, “For centuries, successful immune strategies to survive malaria have been selected in individuals infected by helminths” [347]. This implies
that helminth infection would favor survival until reproductive age in the presence of genetic polymorphisms that protected against cerebral malaria and that malaria may in turn select for genetic predispositions that favor persistence of helminths. It is hypothesised that increased expression of membrane CD23 and increased concentrations of IgE leading to activation of the CD23/NO pathway may be one result of this genetic association [347]. NO production in this setting may also be influenced by intestinal parasite directed Th1-mediated responses as summarised above. Inter-species interactions involving malaria, intestinal helminths and other infectious organisms is an area of research receiving increasing attention and it is likely that hypotheses of this sort will be further refined in the near future [348-350]. Thus, although the evidence is indirect and the hypothesis speculative, intestinal and other infections whose co-existence with malaria may have been selected for in the Papuan population may at least in part explain the higher levels of basal NO production that were seen relative to Darwin controls.

11.4.4. Origins of increased nitric oxide production in Papuan subjects and controls

The lack of correlation between NOS activity and the two complementary measures of NOx production in aparasitemic subjects from Papua suggests that NO production from sources other than PBMCs contributed to the high basal levels of systemic NO production that were observed. In parasitemic subjects, it is likely that the majority of NO production attributable to malaria infection was from non-PBMC sources as NOS activity was no different in subjects with or without parasitemia but systemic NO production in parasitemic subjects was almost double that of aparasitemic subjects.

Sources of NO production in relation to human malaria and other chronic infections are difficult to document due to the difficulty in sampling tissues other than blood, particularly in relation to healthy individuals with asymptomatic infection. In an autopsy study in Malawian children aged 1.5-9 years with severe malaria, widespread staining for NOS2 was evident in the endothelium of a variety of tissues including skeletal muscle; adipocytes; renal tubular cells and pneumocytes, but was not seen in normal tissue collected from Australian controls [351]. Induction of NOS
has also been demonstrated in human vascular endothelial cells in response to the GPI toxin of *P. falciparum* [149]. Resistance against blood stage malaria in mice has been demonstrated to be associated with splenic expression of NOS2 mRNA in response to infection with *P. chabaudi* AS [352], whereas NOS2 expression in the liver correlated with susceptibility in advanced infection. Detection of NOS2 mRNA in the liver of mice infected with *P. vinckei* has also been demonstrated in macrophages as well as in hepatocytes during late stage infection [353]. Similar findings from a recent study have shown that NO production in the blood and/or spleen of mice in vivo was associated with protection against plasmodium infection due to *P. berghei* and *P. chabaudi*, whereas NO production in brain and liver was associated with pathology in the *P. berghei* model [354]. Another study has shown that LPS-induced NOS2 expression in chimeric mice occurs principally in the parenchymal cells of liver, colon and muscle rather than from resident leucocytes [355]. Collectively these findings demonstrate that production of NO secondary to induction of NOS by *Plasmodium* species and other toxins can occur in cells other than PBMCs in mice and in humans, thus providing a potential explanation for the increased NO production seen in malaria-infected Papuan subjects and the lack or correlation between PBMC NOS activity and systemic NO production more generally.

### 11.4.5. Significance of increased nitric oxide production by Papuan adults and parasitemic individuals

In the only previous report of plasma NOx levels in asymptomatic malaria-exposed adults published prior to this study (from coastal PNG), the median plasma NOx of unfasted subjects was 12.6 μmol/L (range 9.5-34.9; n=12; age range 18-40 years) [267]. These levels were lower than in asymptomatic parasitemic children (median plasma NOx 40 μmol/L; mean age 5 years) and were interpreted as suggesting that NO was the likely mediator of tolerance in children but of far less importance in adults. In that study, the parasite status of adults, urban-rural residence and level of malaria exposure were not reported and the number of subjects was small, which in part prompted further exploration of this concept in the present study. Plasma NOx levels in that study were comparable to those of the Darwin controls in the present study and lower than in the Papuan subjects and controls. The median plasma NOx
in Genyem subjects with *P. falciparum* parasitemia in the present study was comparable to that of the Madang children with asymptomatic parasitemia [267]. The relationship between plasma NOx and age in malaria-exposed individuals is further explored in Chapter 12.

The increase in systemic NO production seen in parasitemic subjects in the present study is highly suggestive of a direct link between *P. falciparum* infection and induction of NO production in adults, which until this study had only been demonstrated in children [282] and *in vitro* models [149]. Subsequently it has been shown that even sub-microscopic level *P. falciparum* parasitemia can strongly induce PBMC NOS activity in experimentally infected previously malaria-naïve subjects [300]. This observation in itself does not prove that NO mediates tolerance of parasitemia in adults (or even that it is indicative of a process leading to tolerance) but is at least consistent with that possibility. Although the pyrogenic threshold of adults is uncertain and levels of parasitemia were low in the present study, a number of asymptomatic Genyem subjects were tolerant of parasitemias that have commonly been associated with febrile responses in non-immune individuals [198]. It is also unclear whether the elevated NO production evident in these subjects would provide any protection from malaria severity as this issue was not addressed at all in this study.

It has previously been suggested that NO production in response to the sum total of all stimuli may be “permissive” of malaria parasitemia by down-regulating pro-inflammatory cytokine responses [179], which focuses attention on the reasons for differences in NO production between individuals. Although no longitudinal studies reported in the Medline literature have prospectively studied the relationship between an individual’s capacity to produce NO and subsequent risk or severity of malaria parasitemia, this has been studied retrospectively in Gabonese children recovering from acute malaria [97]. In that study, basal PBMC NOS activity and NO production by cultured PBMCs obtained 4 months or longer following the illness were higher in children who had recovered from mild malaria than in children with prior severe malaria. Differences in basal production of NO such as this may be accounted for by genetic polymorphisms in the NOS2 gene, which have been suggested to correlate disease severity in malaria in addition to functional differences
in NO production (Chapter 13). Protective polymorphisms may undergo positive selection in populations with a long history of exposure to malaria and other infections [356], which may be another explanation for the differences in basal NO production between Papuans and Darwin controls in the current study. The present study and that of the Thai investigators [347] suggests that intestinal parasitosis should also be further considered as a stimulant of increased NO production in malaria-exposed populations.

11.5. Conclusions

The data from this study suggest that *P. falciparum* induces an incremental increase in systemic NO production from non-PBMC sources beyond the increased basal levels evident in residents of Papua province compared to Darwin controls. Factors that may contribute to the increased NO production of Papuans may include genetic determinants selected for by malaria and other infections as well as concomitant intestinal parasitosis, which was not examined directly but was suggested by the extraordinarily high levels of IgE in the rural subjects. The lack of correlation between PBMC NOS activity and systemic measures of NO production suggests that PBMCs are only one source, (and probably not the major source) of NO production in this setting, particularly in relation to that triggered by *P. falciparum* infection. The lack of correlation between IgE levels and NO production may indicate that IgE is a weak inducer of NO production in this setting or that the relationship is not a straightforward one, possibly depending on the presence of IgE immune complexes or reflecting a threshold effect exceeded by the majority. This study indicates that IL-18 may be one mediator of PBMC NOS induction but is less important with respect to total NO production from all sources. The small study size meant that it was difficult to draw meaningful conclusions on the relationship between *P. vivax* and NO production.
Chapter 12. Nitric oxide production in children and adults from Madang, Papua New Guinea

12.1. Introduction

This study was conducted in PNG between February and June 2000 after political events in Indonesia led to cancellation of further studies at the Genyem field site. The main aims of this study were to test the general validity of the results from the Genyem pilot study and to particularly investigate the hypothesis that NO is the major mediator of malarial tolerance and therefore shows the same age-dependence. The study was designed to increase statistical power by recruiting larger numbers than in Genyem but also by using subjects as their own controls in a longitudinal design. This also enabled more detailed examination of the relationship between NO production and parasitemia as the latter could be controlled. The project was borne from the establishment of a collaboration between the MSHR and the PNGIMR. The specific hypotheses to be tested in this project were that:

- Basal systemic NO production shows the same age-dependence as tolerance in highly malaria endemic populations.
- Systemic NO production is triggered by, and proportional to, malaria parasitemia.
- Both *P. falciparum* and *P. vivax* trigger NO production.
- The main source of systemic NO production in highly malaria-endemic populations is circulating mononuclear cells.
- Levels of IgE would be positively correlated with NOS activity in particular and NO production in general.
- Levels of C-reactive protein (CRP), a marker of general inflammation, would be positively correlated with NO production.

These hypotheses were addressed by measuring systemic NOx and circulating mononuclear cell NOS activity cross-sectionally and longitudinally before and after anti-malarial treatment in subjects without clinical malaria from a region where malaria is highly endemic (Madang province, PNG).
12.2. Methods

12.2.1. Study site

PNG lies between 0° and 12° south of the Equator. The north coast climate is tropical with a monsoonal season between October and May. Further inland, the coastal zone rises to highland peaks of over 4500 m elevation and the climate is more temperate. In July 2000, the population reached over 5 million people for the first time [357], with 37% under the age of 15 years and an annualised population growth rate of 2.5% [358]. The infant mortality rate was estimated at 60 per 1000 live births in year 2000, with a life expectancy at birth estimated to be 63 years. Despite severe droughts brought about by the El Nino weather pattern in 1997, economic growth in 1999 was estimated to be 3.6%, with a gross domestic product (GDP) per head of population of US$2500.

The town of Madang lies on the north coast of PNG approximately 5.2° south of the Equator, and is the capital of Madang province which measures approximately 28000 square kilometres in area and has a population of over 360000 [357]. Like elsewhere in PNG, the bulk of the province’s population are subsistence agriculturalists. The subjects recruited into the study were residents of two coastal villages (Haven and Midiba) located approximately 20 km north of Madang town. Extended family units inhabited dwellings constructed of local timbers in village sub-units called hamlets. Homes were not screened against insects, and bed nets, although commonly used, were often in a poor state of repair (personal observation). The predominant source of carbohydrates for study participants were root and other vegetables grown in gardens nearby the village. Limited opportunities exist for the provision of protein, with most families owning one or two pigs, a few chickens and some rudimentary fishing gear. Canned meat is relatively expensive.

12.2.2. Malaria in Madang province

The Madang region is characterised by infection with all four human malaria species and there is little seasonal variation in parasitemia rates [193]. Residents are
estimated to receive on average close to one infective bite per day [359], with an increase in transmission during the wet season from October to May [360]. Parasite prevalence in the region reaches its peak of over 60% in the 5-9 year age group, which is later than that commonly observed from hyperendemic regions in Africa [13,193]. Small area variations in endemicity have been reported within a 22 km radius of Madang town, with the study villages located within the “high epidemiological zone” [359]. Clinical malaria epidemiology in the Madang has been well described in children aged 2 to 15 years and is characterised by a low incidence of severe disease, with episodic mild malaria that decreases in frequency from approximately 5 fever episodes per year at age 2, to 1 per year at age 14 [13]. Severe anemia is more common than cerebral malaria in children [361].

12.2.3. Subjects and controls

Residents from the study villages were notified by the study team on the night before screening was to commence in their hamlet. Non-pregnant adults and children ≥ 1 year of age who volunteered to participate were asked to provide informed consent (additional consent of parents was sought in the case of children), and then screened for enrollment using a clinical questionnaire administered in Tok Pisin (the local language); measurement of axillary temperature and weight; and examination of a fingerprick blood smear for malaria parasites.

Volunteers were excluded from enrollment if they were febrile (axillary temperature ≥ 37.5°C); had taken anti-malarial or non-steroidal anti-inflammatory medication within the past week; had clinical evidence of malaria infection within the past week (any of fever, chills, sweats, headache or myalgia); had a history of infection (other than the common cold) in the past week; or had diarrhea. Those with fever at the time of screening were given treatment for malaria according to revised national guidelines [362] if this diagnosis was confirmed on microscopy, or alternatively taken or referred to other health care providers for treatment of other illnesses.

Between 19 and 44 volunteers were screened per day to enable enrollment of up to 16 subjects per day. Enrollment was selective, with the aim of including subjects with and without parasitemia, and a bias toward *P. falciparum* and *P. vivax* infection.
(and against mixed infection) in parasitemic subjects. A general aim was to include balanced numbers of subjects from different age groups and to include a parent or older sibling of younger children who were enrolled for the convenience of participants.

Sample sizes were calculated prior to the study on the basis of data collected from the previous studies in Papua (Chapter 11) and Tanzania [96]. Based on the assumption that there would be a 30% difference in NO production between parasitemic and aperasitemic subjects, it was calculated that balanced samples of 100 adults and 100 children would be sufficient to show statistically significant differences in a cross-sectional analysis at a level of P<0.05 and a power of 80%. Although difficult to calculate pre-hoc, it was estimated that this would be more than enough subjects to show statistically significant differences longitudinally given the increased power inherent in using subjects as their own controls. In practice, the number of subjects enrolled was influenced by a number of external factors, including time constraints; availability of trained staff from the PNGIMR; numerous minor logistical problems; inclement weather; and higher than expected numbers of subjects meeting the exclusion criteria or having multiple malaria species on combined readings of their two blood smears.

Adult Darwin controls were recruited from amongst the staff and students of MSHR (Chapter 11.2.2.2).

12.2.4. Study protocol

Chicken and rice were distributed to participants on the afternoon of screening, at which time axillary temperature readings were repeated and instructions were given regarding the fasting protocol (Chapter 8.3). Meals were generally prepared in a limited number of houses by boiling the food in water collected from local creeks that had previously been tested and found to be NOx free. One or more members of the study team slept overnight in the villages and were on hand to supervise adherence to the fasting protocol in the evening and the following morning.
Subjects were reviewed the morning after fasting, at which time axillary temperatures were again taken along with collection of a sample of urine and venous blood. Following collection of specimens, subjects with parasitemia on the screening blood smear were given anti-malarial medication to take at doses consistent with revised national PNG guidelines [362]. Subjects with *P. falciparum* ± other parasites were given a single dose of 25 mg of sulfadoxine per kg of body weight and 1.25 mg/kg of pyrimethamine (Fansidar; Roche, NSW). Subjects with parasites not including *P. falciparum* were given 3 daily doses of 10 mg base/kg of chloroquine phosphate (Pharmamed, Malta). Blood smears were repeated on the venous blood specimen, and if *P. falciparum* was seen for the first time on that smear, the subject was given a single dose of sulfadoxine-pyrimethamine to take the following day and advised to cease the chloroquine. As 16 subjects per day were generally enrolled and specimens needed to be processed in the Yagaum laboratory soon after collection, it was not possible to directly supervise the ingestion of medication.

Subjects were reviewed on 2 subsequent occasions as close as possible to 2 weeks and 8 weeks after their initial enrollment, using the same procedures for data and specimen collection as outlined above. For convenience, the notation “T₀” will be used from this point onward to refer to the time-point corresponding with enrollment, and “T₁” and “T₂” to denote the first and second follow-ups respectively.

**12.2.5. Laboratory assays**

Blood smears, plasma and urine NOx, and PBMC NOS activity were assayed as described in Chapter 9. Plasma Cr and plasma IgE were measured using an automated analyser by Ian Gardner of Queensland Medical Laboratories. A diagnostic PCR was used by Joanne Baker at the Army Malaria Institute (Queensland) to detect *P. falciparum* MSP-1 and *P. vivax* apical membrane antigen-1 in blood spotted onto filter paper (Chapter 8.4.1) as previously described [363,364]. The purpose of this PCR was to further increase the certainty that treatment was effective in subjects who were microscopically parasitemic at T₀ and a-parasitemic at T₁.
12.2.6. Statistical methods

Statistical analyses were generally performed and are presented as described in Chapter 10. Parasite densities were grouped into ordered quintiles of approximately equal numbers as the recorded values were unable to be readily normally transformed. These quintiles were cut at 74, 140, 315 and 944 trophozoites/µL in the case of overall parasitemia and at 71, 151, 361 and 1024 trophozoites/µL in the case of *P. falciparum* parasitemia. IgE levels were grouped into ordered quintiles of roughly equal numbers as the recorded values spanned a broad range, were highly skewed and unable to be readily normally transformed. These quintiles were cut at 700, 1800, 2800 and 6000 kU/L. CRP levels were considered on a continuous scale and also as a binary variable (elevated versus normal).

12.3. Results

12.3.1. Baseline characteristics

12.3.1.1. Basic demographics

Demographic surveys recording name, age, gender and place of residence for all individuals living in the villages were conducted prior to the commencement of screening. The first was performed by Moses Lagog prior to my arrival in Madang, which identified 275 residents of 6 hamlets in Haven and included 23 subjects who resided in nearby bush camps. The second survey, conducted under my supervision, identified another 339 residents of 4 hamlets in Midiba and included additional information regarding vocation. The age distribution of the population surveyed (median age 17 years; IQR 7-28) was typical of a developing country with a high fertility rate (Figure 12.1; [365]).
Figure 12.1. Age distribution of PNG subjects

Data was available describing the vocation of 329 (97%) of the 339 residents of Midiba. In children and adolescents aged 5-17 years inclusive: 39% remained in their village during the day; 58% attended school; 1 individual worked outside the village; and no data was available for 3%. In adults aged 18 years and over, 72% stayed in their village during the day, 22% worked outside the village (most females were shift workers at a tuna cannery located midway between the village and Madang town, and most men worked at the Madang timberworks in town), and data was unavailable for 6%. The majority of people who stayed in their villages during the day worked on their gardens, which were located within walking distance.

12.3.1.2. Screening

Seventeen babies < 1 year of age were identified in the demographic surveys who were too young to be enrolled in the study. Time constraints precluded screening 2 hamlets in Haven with a combined population of 45 (Baileb and Limahon; including
This left 554 individuals who were eligible to be screened for enrollment into the study (median age 18 years; IQR 8-29; 53% male). There were no significant differences in age or sex between eligible subjects from the 2 villages.

Single blood smears from 424 individuals aged ≥ 1 year (264 [62%] from Midiba; 48% male) were screened by a single expert microscopist to enable selection of subjects for enrollment. The proportion of subjects overall with malaria parasitemia at screening (45%) was the same in the two villages and reached a peak of 70% in the 5-9 years age group (Figure 12.2). The highest prevalence of *P. falciparum* parasitemia (50%) was found in the 1-4 year age group (Figure 12.2). The prevalence of parasitemia in different age groups; splenomegaly (83% in subjects aged ≤ 14 years with peak of 92% in children 5-9 years); and stated bed net use (86% overall; 98% in children 1-4 years) was broadly consistent with previous data reported from this region [13,193].

![Figure 12.2. Malaria parasitemia prevalence in 424 screened PNG subjects](image-url)

Sixty five percent of the adults from Midiba who were working outside their village were screened, compared to 78% of those who stayed in the village during the day.
(P=0.11). However, following screening, working adults were significantly less likely than those staying in the villages to be enrolled into the study (13% vs 44%; P=0.004).

12.3.1.3. Enrollment

The number of subjects enrolled into the study at $T_0$ totalled 216, of which 127 (59%) were from Midiba and the remainder from Haven (Table 12.1). It is notable that only 2 children of 1 year of age were enrolled in the study. Residents of the bush camps (Chapter 12.3.1.1) had requested to be screened but asked not to be enrolled.

Table 12.1. PNG subjects enrolled in the villages of Haven and Midiba

<table>
<thead>
<tr>
<th>Hamlet</th>
<th>Subjects (no.)</th>
<th>Male (%)</th>
<th>Age in years (median [IQR])</th>
<th>Parasitemic (%)</th>
<th>P. falciparum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haven</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diramiog</td>
<td>25</td>
<td>64</td>
<td>13 (7-20)</td>
<td>88</td>
<td>68</td>
</tr>
<tr>
<td>Haven</td>
<td>16</td>
<td>63</td>
<td>11 (5-13)</td>
<td>69</td>
<td>56</td>
</tr>
<tr>
<td>Hivildig</td>
<td>32</td>
<td>34</td>
<td>20 (14-29)</td>
<td>56</td>
<td>38</td>
</tr>
<tr>
<td>Sablait</td>
<td>16</td>
<td>38</td>
<td>19 (10-26)</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td>89</td>
<td>48</td>
<td>15 (9-25)</td>
<td>69</td>
<td>54</td>
</tr>
<tr>
<td><strong>Midiba</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eregroba</td>
<td>32</td>
<td>50</td>
<td>10 (7-15)</td>
<td>81</td>
<td>63</td>
</tr>
<tr>
<td>Kaironba</td>
<td>31</td>
<td>55</td>
<td>13 (7-28)</td>
<td>74</td>
<td>45</td>
</tr>
<tr>
<td>Mebat</td>
<td>48</td>
<td>35</td>
<td>19 (7-29)</td>
<td>67</td>
<td>50</td>
</tr>
<tr>
<td>Mioruba</td>
<td>16</td>
<td>25</td>
<td>15 (9-20)</td>
<td>56</td>
<td>56</td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td>127</td>
<td>43</td>
<td>12 (7-24)</td>
<td>71</td>
<td>53</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>216</td>
<td>45</td>
<td>13 (7-24)</td>
<td>70</td>
<td>53</td>
</tr>
</tbody>
</table>

Two blood smears taken on consecutive days were available for 198 subjects. In 16 subjects, a second smear was unavailable due to rain washing out collection (below).
In these subjects, treatment was given the following morning and the result from the first smear only was used to classify parasitemia. In subjects who had 2 blood smears available for analysis, one or more additional parasite species were found in 21 of 77 (27%) initially aparasitemic subjects and in 28 of 121 (23%) subjects who were parasitemic on their screening smear. There were no significant differences in age (P=0.10), sex, or proportion of subjects with malaria parasitemia (or parasitemia with *P. falciparum*) between villages.

The baseline characteristics of the adult Darwin controls were described in Chapter 11.3.1.3.

### 12.3.1.4. Specimen availability

Heavy rain that prohibited travel to the study site forced the cancellation of specimen collection in 16 subjects at T₀. Blood was otherwise collected from 99.4%, 96% and 98% of included subjects at T₀, T₁ and T₂ respectively. Urine was collected from 98%, 91% and 95% of included subjects at T₀, T₁ and T₂ respectively. The reasons for unavailability of specimens included difficulty in venesecting young children and inability of some subjects to produce a urine specimen within the time constraints relating to collection.

### 12.3.1.5. Follow-up

Follow-up at T₁ was attempted for all subjects and completed for 193 (89%) of the 216 enrolled subjects after a median of 14 days (range 12-32 days). Of these, 147 (76%) were followed up on the 14ᵗʰ day and 27 (14%) on the 28ᵗʰ day. There were no significant differences in age, gender or village of origin in the 23 subjects who left the study after enrollment compared to those who were followed up at T₁. While it was not possible to determine the exact reason subjects dropped out of the study, on a number of occasions family members mentioned that their kin were unavailable for follow up due to travel or work (personal observation).

Due to time constraints, it was not possible to conduct a second follow up at T₂ in 40 subjects who were enrolled late in the study. Another 41 subjects from 2 hamlets
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(Hvildig and Mioruba) were also unavailable as they and their leaders had agreed to participate on the basis that they be followed up only once. Eight (7%) of the remaining 112 subjects from T\textsubscript{1} did not attend follow up at T\textsubscript{2}. Two subjects who had dropped out of the study at T\textsubscript{1} re-entered at T\textsubscript{2}, meaning that overall, 106 subjects were followed up at T\textsubscript{2} after a median of 56 days from T\textsubscript{0} (range 40-77).

12.3.1.6. Treatment, clearance and recurrence of parasitemia

Antimalarial medication was given to 148 of 151 parasitemic subjects with the intention of eradicating parasitemia. Three subjects did not receive antimalarials as their parasitemia was only detected on subsequent cross-checking of blood smears. Sulfadoxine-pyrimethamine alone was given to 100 (66%) of the parasitemic subjects and chloroquine alone to 34 (23%). Both sulfadoxine-pyrimethamine and chloroquine were given to 14 subjects (9%), in whom \textit{P. falciparum} was only seen on the second consecutive smear after chloroquine had been given on the basis of the result from the first smear. One subject who was given chloroquine treatment later turned out to have no parasites seen on cross-checking of their blood smears.

Treatment was successful in eradicating parasites completely in 121 (87%) of the 139 initially parasitemic subjects who attended follow up at T\textsubscript{1} and in 90 (87%) of the 104 subjects whose baseline parasitemia included \textit{P. falciparum}. \textit{P. falciparum} parasitemia persisted in 9 of the 14 subjects with \textit{P. falciparum} at T\textsubscript{0} who were still parasitemic at T\textsubscript{1} (i.e., the success rate for clearing \textit{P. falciparum} was 91%). The \textit{P. falciparum} PCR was positive in 13 (65%) of 20 microscopically positive samples tested to validate the PCR, and the \textit{P. vivax} PCR was positive in 9 (75%) of 12 microscopically positive samples. PCR was done at T\textsubscript{1} for 99 of 100 subjects who were: parasitemic at T\textsubscript{0}; given treatment; aparasitemic at T\textsubscript{1}; and not excluded at either time-point. Only 4 of these 99 samples were positive for parasites by PCR, 3 for \textit{P. falciparum} and 1 for \textit{P. vivax}. Two (2.6%) of 77 subjects with \textit{P. falciparum} who were treated at T\textsubscript{0} and aparasitemic at T\textsubscript{1} were PCR positive for \textit{P. falciparum} and no subjects with \textit{P. vivax} at T\textsubscript{0} were PCR positive for \textit{P. vivax} at T\textsubscript{1}. In contrast, in a random selection of 32 microscopically aparasitemic subjects from T\textsubscript{0}, 8 (25%) were positive for \textit{P. falciparum} and 1 (3%) for \textit{P. vivax}. 

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Seventy four subjects were followed up at T2 who had been given treatment to eradicate parasitemia at T0 and who had become aparasitemic on microscopy at T1 (median age 11 years; IQR 7-18). Parasitemia recurred in 38 (47%) of these subjects after a median of 42 days from T1 (IQR 30-63), with *P. falciparum* present in 18 (47%) and *P. vivax* in 21 (60%). *P. falciparum* parasitemia had recurred in 12 (21%) of 57 subjects who had been given treatment to clear infection at T0. The likelihood of parasite recurrence (any parasite) was strongly influenced by the number of days that had elapsed between T1 and T2 (Figure 12.3; adjusted for age group) and the age of subjects (Figure 12.4; adjusted for time). For each week that elapsed after T1, the likelihood of recurrence increased 1.9 times (95% CI 1.3-2.6; P<0.005), and for each successive age group, the likelihood of recurrence was 0.5 times that of the previous age group (95% CI 0.3-0.8; P=0.006). There was no relationship between the likelihood of recurrence and any measure of NO production at baseline (T0).

![Graph](image)

**Figure 12.3.** Predicted probability (95% CI) of recurrence of parasitemia at T2 as a function of the number of weeks elapsing between T1 and T2.

Subjects were those in whom parasitemia had been eradicated with anti-malarial treatment at T0 (confirmed on microscopy at T1).
Figure 12.4. Predicted probability (95% CI) of recurrence of parasitemia at $T_2$ by age group

Subjects were those in whom parasitemia had been eradicated with anti-malarial treatment at $T_0$ (confirmed on microscopy at $T_1$).

12.3.1.7. Inclusion and exclusion of subjects

Three axillary temperatures were taken at $T_0$ in 170 subjects (79%); 2 in 30 subjects (14%) and 1 only in the 16 subjects whose initial enrollment was rained out after the first morning (Chapter 12.3.1.4). At $T_1$, 3 axillary temperatures were taken in 139 subjects (72%); 2 in 48 (25%) and 1 only in 6 (3%). At $T_2$, 3 axillary temperatures were taken in 75 subjects (71%); 2 in 28 (26%) and 1 only in 3 (3%). Seven of the 12 subjects who were febrile during the study also met other exclusion criteria, therefore it was decided that subjects with only 1 or 2 temperature readings available who were not febrile would not be excluded from the analysis.

Overall, 38 subjects (18%) met the exclusion criteria at one or more time-points (Table 12.2) and were excluded from subsequent statistical analyses involving data
collected at those times. There was no significant difference in the proportion of subjects meeting the exclusion criteria at any of the 3 time-points.

Table 12.2. Reasons for PNG subjects meeting exclusion criteria

<table>
<thead>
<tr>
<th>Time-point</th>
<th>Subjects</th>
<th>Exclusions</th>
<th>Illness²</th>
<th>Medicine³</th>
<th>Diarrhea</th>
<th>Febrile⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>216</td>
<td>21 (10)</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>T₁</td>
<td>193</td>
<td>15 (8)</td>
<td>10</td>
<td>7</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>T₂</td>
<td>106</td>
<td>9 (8)</td>
<td></td>
<td>7</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

¹Totals may exceed the number of subjects due to one subject being excluded at more than one time-point or for more than one reason. ²History of fever or malarial symptoms in previous week. ³History of ingestion of anti-malarial medication or non-steroidal anti-inflammatory drug within previous week. ⁴Measured axillary temperature of = 37.5°C on either of 3 occasions over 24 hours during enrollment.

The baseline characteristics for the 195 subjects included in the statistical analysis at T₀ are given in (Table 12.3). The median age of the subjects aged = 20 years was 30 years (IQR 24-35). Parasitemia with any single malaria species, or combination of species, was present in 69% of subjects overall and with P. falciparum in 54%.
Table 12.3. Baseline characteristics of parasitemias in PNG subjects included in the statistical analysis

<table>
<thead>
<tr>
<th>Agegp. (years)</th>
<th>n</th>
<th>Male</th>
<th>%</th>
<th>Pf</th>
<th>Pf/Pv</th>
<th>Pf/Pm</th>
<th>Pf/Pv/Po</th>
<th>Pv</th>
<th>Pm</th>
<th>Po</th>
<th>Neg (%)</th>
<th>Pt (no. [%])</th>
<th>Pf/μL (95% CI)</th>
<th>Parasites/μL (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-4</td>
<td>22</td>
<td>32</td>
<td>32</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>5 (23)</td>
<td>14 (64)</td>
<td>1008 (403-2520)</td>
<td>1267 (623-2577)</td>
</tr>
<tr>
<td>5-9</td>
<td>38</td>
<td>53</td>
<td>53</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>6 (16)</td>
<td>27 (57)</td>
<td>429 (219-839)</td>
<td>453 (267-780)</td>
</tr>
<tr>
<td>10-14</td>
<td>39</td>
<td>51</td>
<td>51</td>
<td>6</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>7 (18)</td>
<td>23 (33)</td>
<td>323 (176-592)</td>
<td>259 (156-430)</td>
</tr>
<tr>
<td>15-19</td>
<td>23</td>
<td>39</td>
<td>39</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>7 (30)</td>
<td>16 (70)</td>
<td>163 (90-295)</td>
<td>191 (111-327)</td>
</tr>
<tr>
<td>20+</td>
<td>73</td>
<td>45</td>
<td>45</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>2</td>
<td>1</td>
<td>35 (48)</td>
<td>25 (34)</td>
<td>107 (68-168)</td>
<td>95 (69-132)</td>
</tr>
<tr>
<td>Total</td>
<td>195</td>
<td>46</td>
<td>46</td>
<td>21</td>
<td>21</td>
<td>8</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>20</td>
<td>60 (31)</td>
<td>105 (54)</td>
<td>280 (207-379)</td>
<td>262 (202-339)</td>
</tr>
</tbody>
</table>

1 Number of subjects with each species (or combined species) of parasite on examination of two consecutive daily blood smears. Pf = P. falciparum; Pv = P. vivax; Pm = P. malariae; Po = P. ovale; and Neg = negative for parasites. 2 Number and percentage of subjects with P. falciparum parasitemia either as the sole infecting parasite or in combination with other parasites. 3 Geometric mean of the highest-density P. falciparum parasitemia or the highest densities of combined species measured from two consecutive daily blood smears.
12.3.2. Renal function and fractional excretion of NOx

12.3.2.1. Children and adolescents aged 2-18 years

Plasma Cr was normally distributed and independently related to both age and weight (but not sex) in children and adolescents aged = 18 years ($r^2=0.65; P<0.001$). The mean plasma Cr and the percentage of children in each 3 year age group with a plasma Cr below the age-specific reference range is presented in Table 12.4. No child had a plasma Cr greater than the middle value of their age-specific reference range. Ten subjects aged = 18 years had proteinuria present on urine dipstick analysis on at least one occasion but none of these children had proteinuria on repeated urinalysis. The percentage of children whose weight was below the median standardised weight for age [366] and less than 2 SDs from this median is also presented in Table 12.4. The median FENOx in subjects = 18 years was 36% (IQR 28-46%) and is presented for each of the 3 year age subgroups in Table 12.4. There were no significant age or sex related differences in FENOx in this age group.
Table 12.4. Renal function and fractional excretion of NOx in PNG children and adolescents

<table>
<thead>
<tr>
<th>Age gp.</th>
<th>n</th>
<th>(N range for age)</th>
<th>Mean Cr (95% CI)</th>
<th>Cr&lt;sup&gt;1&lt;/sup&gt;</th>
<th>% &lt; N Cr&lt;sup&gt;1&lt;/sup&gt;</th>
<th>n &lt; med. Weight&lt;sup&gt;3&lt;/sup&gt;</th>
<th>n &lt; 2×SD below med. Weight&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Med. FENOx (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>14</td>
<td>35-62</td>
<td>31 (27-35)</td>
<td>86</td>
<td>12</td>
<td>3</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>4-6</td>
<td>15</td>
<td>44-71</td>
<td>38 (34-41)</td>
<td>93</td>
<td>14</td>
<td>3</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>7-9</td>
<td>23</td>
<td>53-88</td>
<td>42 (39-46)</td>
<td>96</td>
<td>21</td>
<td>7</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>10-12</td>
<td>23</td>
<td>53-88</td>
<td>46 (44-49)</td>
<td>87</td>
<td>23</td>
<td>13</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>13-15</td>
<td>22</td>
<td>53-106</td>
<td>53 (49-56)</td>
<td>59</td>
<td>22</td>
<td>14</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>16-18</td>
<td>10</td>
<td>71-123</td>
<td>60 (54-66)</td>
<td>90</td>
<td>10</td>
<td>3</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td>45 (43-47)</td>
<td>84</td>
<td>102</td>
<td>43</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Normal plasma creatinine range for age group as used by Royal Darwin Hospital.
<sup>2</sup>Percentage of children with a plasma creatinine below the normal range.
<sup>3</sup>Number of children whose weight was less than the median age-standardised weights as defined by the US National Center for Health Statistics and awaiting re-ratification by the World Health Organization.
<sup>4</sup>Number of children whose weight was less than 2 standard deviations below the standardised median weight for age.

12.3.2.2. Adults = 19 years

There was no significant difference in the median ages of males (30 years; IQR 23-35) and females (27 years; IQR 24-32) for whom a plasma Cr result was available in this age group. Plasma Cr was normally distributed in both sexes and within the normal ranges (males: 60-120 µmol/L; females: 50-110 µmol/L) for 35 of the 36 females and 33 of the 35 males. One female had a plasma Cr below the normal range (47 µmol/L) and two males had a plasma Cr of 125 µmol/L. The mean plasma Cr was significantly higher in males (97 µmol/L; SD 13) than in females (70 µmol/L; SD 15; P<0.001) and was very close to what had been previously reported in healthy males and females from coastal Madang (males 100 µmol/L; females 68 µmol/L; [367]). In a bivariate model, predicted plasma Cr was 26 µmol/L (95% CI 20-33; P<0.001) higher in males than in females and each 5 years of age was associated with a predicted 1.9 µmol/L (95% CI 0.3-3.5; P=0.019) increase in plasma Cr. Plasma Cr was not independently associated with body weight. Proteinuria was
present on at least one dipstick analysis in 11 (14%) of 76 subjects aged = 19 years. Eight of these 11 subjects had repeated urinalyses performed which were negative for proteinuria, and 3 of these subjects produced only 1 urine sample during the study.

The calculated GFR was normally distributed in these adults (mean 82 mL/min; SD 20) and there was no significant difference in GFR between males and females. The calculated GFR was below the normal range of 80-120 mL/min (standardised against a BSA of 1.73 m$^2$) in 27 (38%) of the 71 subjects. The median FENOx was 34% (IQR 28-49) in adults and did not differ significantly between males and females.

12.3.3. Cross-sectional analysis at $T_0$

12.3.3.1. Baseline nitric oxide production

12.3.3.1.1. Urine NOx excretion

Baseline urine NOx/Cr results were available for 194 (90%) of the 216 enrolled subjects (Chapter 12.3.1.4). Results from a further 23 subjects were excluded from statistical analysis due to UTI (2 subjects), subject meeting general exclusion criteria (20 subjects) or both (1 subject). The median age of the remaining 171 subjects considered in the statistical analysis was 15 years (IQR 9-24; range 1-57).

The median urine NOx/Cr in the 171 subjects meeting the inclusion criteria in whom results were available was 0.22 (IQR 0.15-0.39). The unadjusted relationship between urine NOx/Cr and age in years is shown in Figure 12.5 and summarised by age groups in Figure 12.6. Urine NOx/Cr was negatively associated with increasing age in years ($r^2=0.22; P<0.001$) but appeared to plateau in subjects $\geq$ 20 years (Figure 12.5). The predictive power of the regression model was highest when age was considered in ordinal groups (1-4, 5-9, 10-14, 15-19 and 20+ years; $r^2=0.35; P<0.001$).
Figure 12.5. Relationship between urine NOx/Cr and age at $T_0$

Dots represent the ratio of urinary NOx (μmol/L) to Cr (μmol/L). The medians of bands of approximately 10 observations each are joined by a cubic spline (line).
Figure 12.6. Relationship between urine NOx/Cr and age group at T₀

The decrease in urine NOx/Cr with age in subjects = 19 years may theoretically have reflected a decrease in overall NO production, an increase in urine Cr excretion, or both. Twenty four hour urinary excretion of Cr has been shown to increase with age in healthy children and adolescents from Japan [368] and Germany [369] and has been shown to strongly correlate with both the children’s height and weight [369]. This is likely to reflect the relationship between muscle mass and Cr production [370]. Differences in urine Cr excretion in the PNG cohort may potentially have resulted in a decrease in the urine NOx/Cr ratio with age due to an increase in the denominator used to calculate the ratio [371].

Because the ratio of 24 hour urinary excretion of an analyte to that of Cr can be approximated by the ratio in spot concentrations of the two [372], the daily excretion of the analyte can be estimated by multiplying the spot ratio by the 24 hour urinary Cr excretion. Reference values of 24 hour urinary Cr excretion have been derived from measured values in healthy German children aged 3-18 years and standardised according to weight in separate tables for males and females [369]. Due to the absence of similar data from PNG, the urine NOx/Cr ratios in 91 PNG children aged
3 to 18 years were multiplied by the weight-standardised reference values from the German children to estimate the 24 hour urinary NOx excretion (Figure 12.7). In contrast to the relationship between the spot urine NOx/Cr ratio and age in subjects < 20 years, age was associated with a significant increase in the predicted NOx excretion in the subset of children aged 3-18 years ($r^2=0.11; P=0.001$). For every 3 year increment in age, NOx excretion was predicted to increase by 16% (95% CI 6-27). There was no significant difference in predicted NOx excretion between male and female subjects.

![Figure 12.7. Estimated 24 hour urine NOx excretion (mmol) for PNG children and adolescents](image)

There was no significant relationship between urine NOx/Cr and age, gender, weight or plasma Cr in PNG subjects aged = 19 years. The GM urine NOx/Cr ratio in PNG subjects = 19 years (0.15; 95% CI 0.13-0.17) was 3 times higher than Darwin adult controls (0.05; 95% CI 0.04-0.06; P<0.001) and this was not affected by adjusting for the non-significant difference between the groups in age. NOx excretion in the PNG adults was estimated as above using weight-standardised 24 hour urinary Cr excretion values from the healthy German children [369] as these encompassed the
full range of adults’ weights from PNG. When calculated in this way, NOx excretion was still not significantly associated with age or sex in subjects = 19 years. The relationship between estimated NOx excretion (mmol/day) and age for subjects = 3 years is shown in Figure 12.8.

![Figure 12.8. Estimated 24 hour urine NOx excretion for all PNG subjects = 3 years](image)

Dots represent individual values from 160 subjects. The medians of bands of 10 observations each are joined by a cubic spline (line).

Estimated 24 hour NOx excretion was not calculated in Darwin controls as the weight-standardised urinary Cr excretion values from the German children did not cover the full range of Darwin subjects’ weights.

12.3.3.1.2. Plasma NOx

Baseline plasma NOx results were available for 199 of the 216 enrolled subjects (Chapter 12.3.1.4). Results from the 21 subjects who met the exclusion criteria at T₀
were excluded from the statistical analysis. The median age of the remaining 178 subjects was 14.5 years (IQR 9-24; range 1-60).

The median plasma NOx in these 178 subjects was 37 µmol/L (IQR 25-55) compared to 11 µmol/L (IQR 7-24) in Darwin controls. Plasma NOx was significantly lower in Darwin controls than in each age group of PNG subjects (P<0.001 in each case). The relationship between plasma NOx and age in years in PNG subjects is shown in Figure 12.9 and summarised by age groups (with the inclusion of Darwin controls) in Figure 12.10. Plasma NOx in PNG subjects varied significantly amongst age groups (P=0.017), but although plasma NOx was highest in the youngest children and lowest in adults (P=0.007), there was no consistent age-related trend. This was reflected in a linear regression model showing that although significant, age only explained 2.5% of the overall variation in plasma NOx (P=0.033). In this model, plasma NOx was predicted to fall by 3.5% for every 5 years of age, from a high of 41 µmol/L at age 2, to 36 µmol/L at age 20, and 31 µmol/L at age 40. There were no sex related differences in plasma NOx at any age and there was no significant relationship between plasma NOx, plasma Cr or calculated GFR (in adults only).
Figure 12.9. Plasma NOx in PNG subjects
Dots represent plasma individual values (µmol/L). The medians of bands of approximately 10 observations each are joined by a cubic spline (line).
Baseline results for the measurement of PBMC NOS activity were available from 158 subjects (blood was unavailable from 17 subjects as discussed in Chapter 12.3.3.1.4; 21 subjects were excluded from the analysis; 12 samples went astray during transport from PNG to Australia; and a further 11 went astray during transport to the US). The median age of the 158 subjects for whom a PBMC specimen was available and who were not excluded from the statistical analysis was 15 years (IQR 9-24; range 1-57).

The median PBMC NOS activity in these 158 subjects was 1031 pmol/mg (IQR 669-1535; range 110-3665). The relationship between NOS activity and age is shown in Figure 12.11 and summarised by age group in Figure 12.12. In contrast to urine NOx/Cr and plasma NOx, there was no significant association between PBMC NOS activity and age in different regression and ANOVA models considering age as a linear variable, or in ordinal or categorical age groups. PBMC NOS activity was
significantly higher in PNG subjects than in Darwin controls (median 613 pmol/mg/hr; IQR 495-899; P=0.003).

Figure 12.11. PBMC NOS activity in PNG subjects
12.3.3.1.4. Correlation between different measures of NO production

The two different measures of urine NOx excretion (the urine NOx/Cr ratio and the estimated 24 hour NOx excretion) were better correlated in subjects = 19 years (r=0.94, P<0.001) than in subjects = 18 years (r=0.73, P<0.001). In PNG subjects = 18 years, plasma NOx was significantly correlated with both urine NOx/Cr (r=0.69; P<0.001) and estimated 24 hour urinary NOx excretion (r=0.70, P<0.001). In PNG adults = 19 years, plasma NOx was better correlated with 24 hour NOx excretion (r=0.77, P<0.001) than it was with urine NOx/Cr (r=0.69, P<0.001). Plasma NOx and urine NOx/Cr were significantly correlated in the 22 Darwin controls (r=0.47, P=0.03). This relationship was stronger after excluding the outlying Darwin control with a calculated FENOx of 171% (Chapter 11.3.2.3) from the analysis (r=0.63, P=0.002).

NOS activity was not correlated with any of the urine or plasma measures of NO production in children and adolescents or in adults (r=0.1 and P>0.3 for all comparisons).
12.3.3.2. NO production and parasitemia

Comparing subjects with malaria parasitemia to apanasitemic subjects, there was no association between any measure of NO production and parasitemia in univariate analyses or after adjustment for age group and/or sex at T₀. Likewise, there was no difference in any measure of NO production between subjects with *P. falciparum* parasitemia on blood film and subjects without *P. falciparum* (i.e., apanasitemic subjects and those with other parasites), or those with *P. falciparum* parasitemia and apanasitemic subjects. There was no relationship between the highest recorded density of parasitemia over 2 consecutive days (all parasites) or density of *P. falciparum* parasitemia and any measure of NO production cross-sectionally at T₀. Similarly, there was no significant relationship between the level of parasitemia (both measures) on the day of venesection and any measure of NO production.

12.3.3.3. NO production and IgE

Baseline IgE levels ranged from 35 to 18400 international units (kU/L) and were highly skewed (mean 3606, median 2200 [IQR 1000-4800]). IgE levels were lowest in the youngest age group and increased to a peak in the 10-14 year age group, before decreasing and levelling out in subjects aged 15 years and older (Figure 12.13). IgE levels were significantly predictive of PBMC NOS activity but only explained 4% of the overall variance (P=0.014). Predicted NOS activity was 9% higher in each successive quintile of IgE (95% CI 2-17%). Controlling for the non-significant variables age and sex made no difference to the magnitude or significance of this relationship. There was no association between IgE levels and other measures of NO production. IgE levels were not associated with the presence or density of malaria parasitemia in general or *P. falciparum* in particular.
12.3.3.4. NO production and CRP

CRP levels were elevated above the normal range (>6 mg/L) in 15% of parasitemic subjects at T₀ compared to 0% of aparasitemic subjects (P=0.002). The median CRP level in those subjects in whom it was elevated was 12 mg/L (IQR 10-24). In parasitemic subjects, the CRP level was strongly correlated with density of parasitemia, both overall (Spearman’s r=0.40; P<0.001) and in the sub-group of subjects whose CRP was ≥ 6 mg/L (r=0.25; P=0.009). CRP was just as strongly correlated with P. falciparum density in the sub-group with P. falciparum parasitemia (r=0.42; P<0.001), but the correlation with density in 28 subjects with parasites not including P. falciparum was not statistically significant (r=0.34; P=0.077). The effect of age on CRP levels was not tested in parasitemic subjects as the CRP data could not be normally transformed and levels of parasitemia were strongly related to age, however, CRP and age were not correlated in 51 subjects without parasitemia (Spearman’s rank test).
Subjects with an elevated CRP had a GM plasma NOx that was 30% (95% CI 8-46) lower than subjects with a normal CRP (P=0.012) after controlling for the small but significant effect of age group (Chapter 12.3.3.1.2) on plasma NOx. However, there was no significant linear association between plasma NOx and CRP (including values within the normal range). Elevated CRP was not significantly associated with either NOS activity, urine NOx/Cr or estimated 24 hour NOx excretion.

12.3.3.5. The problem of “kus”

The common cold (known locally in PNG as “kus”) unexpectedly afflicted 2%, 9% and 19% of enrolled subjects not meeting the exclusion criteria at T₀, T₁ and T₂ respectively. The effect of kus on NO production was examined cross-sectionally at these 3 time-points. After adjusting for age group, none of the aforementioned indices of NO production differed significantly in subjects with or without kus at any of these time-points. In paired longitudinal analyses comparing T₀ to T₁, and T₁ to T₂, there were no differences in any index of NO production in subjects who had kus at one time-point but not another.

12.3.4. Longitudinal analysis

There was a high level of consistency in the central tendencies and spread of results between T₀, T₁ and T₂ for all measured indices of NO production (Figure 12.14, Figure 12.15 and Figure 12.16). Within individuals, there was no significant change between results taken at two or more time-points for urine NOx/Cr, plasma NOx or NOS (P>0.20 for each longitudinal ANOVA analysis).
Figure 12.14. Longitudinal urine NOx/Cr in PNG subjects not excluded at any time-point

Dots represent individual values, lines of pluses are the mean and horizontal lines represent standard deviations.
Figure 12.15. Longitudinal plasma NOx in PNG subjects not excluded at any time-point

Dots represent individual values, lines of pluses are the mean and horizontal lines represent standard deviations.
Figure 12.16. Longitudinal PBMC NOS activity in PNG subjects not excluded at any time-point

Dots represent individual values, lines of pluses are the mean and horizontal lines represent standard deviations.

12.3.4.1. Effect of clearance of parasitemia on nitric oxide production

The effect of clearance of parasitemia on NO production was specifically examined in subjects who: were parasitemic for any malaria parasite or combination of parasites on microscopy at T₀; received treatment to clear their parasitemia; were aparasitemic at T₁ and were not excluded at either time-point. There was no significant change in NO production for any of the 3 measured indices between T₀ and T₁ in these subjects (Table 12.5). Results were similar when the analyses were repeated in each age subgroup.
Table 12.5. Effect of clearance of parasitemia on NO production in PNG subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Age in years (med. [IQR])</th>
<th>Baseline result (med. [IQR])</th>
<th>Change T₀ to T₁ (med. [IQR])</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma NOx (µmol/L)</td>
<td>90</td>
<td>13 (9-23)</td>
<td>37 (28-56)</td>
<td>1.2 (-9.4-14)</td>
<td>0.46</td>
</tr>
<tr>
<td>Urine NOx/Cr</td>
<td>84</td>
<td>13 (9-22)</td>
<td>0.24 (0.15-0.37)</td>
<td>0.006 (-0.08-0.12)</td>
<td>0.16</td>
</tr>
<tr>
<td>NOS activity (pmol/mg/hr)</td>
<td>80</td>
<td>13 (9-23)</td>
<td>1046 (673-1519)</td>
<td>-10 (-56-57)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

1Significance of paired Wilcoxon Test

There was no significant change in any measure of NO production in the subgroup of subjects whose initial parasitemia was due to *P. falciparum* (either as the single infecting species or in combination with other species), nor in subjects whose initial parasitemia was due to parasite(s) other than *P. falciparum* (Table 12.6). Results were similar when the analyses were repeated in each age subgroup.

Table 12.6. Effect of clearance of *P. falciparum* or other parasites on NO production in PNG subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Age in years (med. [IQR])</th>
<th>Baseline result (med. [IQR])</th>
<th>Change T₀ to T₁ (med. [IQR])</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma NOx (µmol/L)</td>
<td>70</td>
<td>13 (7-23)</td>
<td>39 (30-57)</td>
<td>-0.03 (-11-12)</td>
<td>1.00</td>
</tr>
<tr>
<td>Urine NOx/Cr</td>
<td>65</td>
<td>13 (9-22)</td>
<td>0.24 (0.15-0.38)</td>
<td>0.005 (-0.07-0.10)</td>
<td>0.21</td>
</tr>
<tr>
<td>NOS activity (pmol/mg/hr)</td>
<td>61</td>
<td>13 (8-23)</td>
<td>1080 (673-1468)</td>
<td>-14 (-57-52)</td>
<td>0.31</td>
</tr>
<tr>
<td><em>Other parasites</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma NOx (µmol/L)</td>
<td>20</td>
<td>12.5 (10-23)</td>
<td>29 (24-40)</td>
<td>4 (-1.2-29)</td>
<td>0.12</td>
</tr>
<tr>
<td>Urine NOx/Cr</td>
<td>19</td>
<td>12 (10-23)</td>
<td>0.23 (0.12-0.30)</td>
<td>0.02 (-0.09-0.15)</td>
<td>0.65</td>
</tr>
<tr>
<td>NOS activity (pmol/mg/hr)</td>
<td>19</td>
<td>13 (10-23)</td>
<td>923 (606-1628)</td>
<td>8.6 (-15-88)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

1Significance of paired Wilcoxon Test
12.3.4.2. **Effect of recurrence of parasitemia on nitric oxide production**

The effect of recurrence of parasitemia on NO production was specifically examined in subjects who: were parasitemic for any malaria parasite or combination of parasites on microscopy at $T_0$; received treatment to clear their parasitemia and were aparasitemic at $T_1$; had recurrent parasitemia at $T_2$ and were not excluded at either $T_1$ or $T_2$. There was no significant change in NO production for any of the 3 measured indices between $T_1$ and $T_2$ in these subjects (Table 12.7).

### Table 12.7. Effect of recurrence of parasitemia on NO production in PNG subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$n$</th>
<th>Age in years (med. [IQR])</th>
<th>$T_1$ result (med. [IQR])</th>
<th>Change $T_1$ to $T_2$ (med. [IQR])</th>
<th>$P$ value $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma NOx ($\mu$mol/L)</td>
<td>31</td>
<td>10 (5-13)</td>
<td>40 (26-59)</td>
<td>2 (-16-22)</td>
<td>0.28</td>
</tr>
<tr>
<td>Urine NOx/Cr</td>
<td>30</td>
<td>6 (10-13)</td>
<td>0.37 (0.21-0.55)</td>
<td>-0.04 (-0.16-0.06)</td>
<td>0.58</td>
</tr>
<tr>
<td>NOS activity (pmol/mg/hr)</td>
<td>30</td>
<td>9.5 (5-13)</td>
<td>1375 (1000-1771)</td>
<td>-19 (-67-59)</td>
<td>0.58</td>
</tr>
</tbody>
</table>

$^1$Significance of paired Wilcoxon Test

There was no significant change in any measure of NO production in the subgroup of subjects whose recurrence of parasitemia included *P. falciparum* (either as the single infecting species or in combination with other species), nor in those whose recurrence of parasitemia was due to parasite(s) other than *P. falciparum* [Table 12.8]).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Age in years (med. [IQR])</th>
<th>T₁ result (med. [IQR])</th>
<th>Change T₁ to T₂ (med. [IQR])</th>
<th>P value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. falciparum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma NOx (µmol/L)</td>
<td>14</td>
<td>11 (7-17)</td>
<td>39 (26-53)</td>
<td>11 (-14-23)</td>
<td>0.79</td>
</tr>
<tr>
<td>Urine NOx/Cr</td>
<td>14</td>
<td>11 (7-17)</td>
<td>0.35 (0.20-0.49)</td>
<td>-0.07 (-0.15-0.06)</td>
<td>0.79</td>
</tr>
<tr>
<td>NOS activity (pmol/mg/hr)</td>
<td>14</td>
<td>11 (7-17)</td>
<td>1388 (1019-2219)</td>
<td>-34 (-271-59)</td>
<td>0.79</td>
</tr>
<tr>
<td><strong>Other parasites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma NOx (µmol/L)</td>
<td>16</td>
<td>7 (3-11)</td>
<td>43 (27-62)</td>
<td>-1.1 (-16-12)</td>
<td>0.46</td>
</tr>
<tr>
<td>Urine NOx/Cr</td>
<td>15</td>
<td>7 (3-10)</td>
<td>0.42 (0.21-0.76)</td>
<td>-0.03 (-0.31-0.05)</td>
<td>0.61</td>
</tr>
<tr>
<td>NOS activity (pmol/mg/hr)</td>
<td>15</td>
<td>7 (3-10)</td>
<td>1319 (647-1715)</td>
<td>-19 (-57-60)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

¹Significance of paired Wilcoxon Test

**12.4. Discussion**

The present study was designed so that basal NO production could be assessed in malaria-exposed subjects in a research environment that was controlled as much as possible. Conducting the study in the field was less disruptive to the lifestyles of participants and was associated with high follow-up rates. Subjects with illnesses or other factors that could potentially influence NO production were rigorously excluded to ensure validity of the statistical analyses. Microscopic parasitemia was defined as accurately as possible by examining 2 slides from most subjects taken on consecutive days and cross-checking slides in a detailed cross-checking procedure. This approach appeared valid as approximately half the subjects had a new or different parasite seen on the second consecutive smear. The major strength of the current study was that the effect of parasitemia on NO production was able to be examined longitudinally, using subjects as their own controls and thereby enhancing statistical power. This also enabled for differences in age to be controlled for as well as other factors that may vary between individuals in terms of stimuli to NO production.
12.4.1. Interpretation of NOx in relation to renal function and renal handling

It has previously been demonstrated that renal dysfunction may falsely elevate plasma NOx and influence the interpretation of urine NOx excretion, as the majority of NOx is excreted renally [96,100,373]. It was therefore important to carefully assess renal function in the study cohort prior to analysis of results. Plasma Cr was normally distributed in adults and only one subject aged = 19 years had a plasma Cr above the reference range, yet 38% had a calculated GFR that was below the normal range. Did this high incidence of decreased GFRs despite normal plasma Cr indicate a high incidence of “covert” renal dysfunction [374]? GFRs calculated by the Cockcroft-Gault equation should be indexed to a BSA of 1.73 m$^2$, which leads to an increased value in subjects whose BSA is < 1.73 m$^2$. Height was not recorded in subjects from the present study but mean heights of coastal people from PNG have previously been reported at 163 cm in males and 153 cm in females [324], which would yield BSAs of 1.60 m$^2$ and 1.43 m$^2$ respectively if applied to the mean weights of males and females in the present study [325]. Baseline GFRs (measured by 24 hour Cr clearance) have been shown to be significantly higher in meat-eaters than in vegetarians [375], which is thought to reflect utilisation of renal reserve for handling protein loads rather than renal dysfunction in vegetarians [376]. A mostly vegetarian diet was the norm in the study villages (personal observation). It is therefore very likely that the GFRs calculated in these subjects would have under-estimated their true GFRs, which may have been lower than the normal range anyway due to protein malnutrition rather than renal failure. Persistent proteinuria, a sensitive early marker of renal dysfunction [374], was not detected in any adults who had repeated urine measures and was only detected in a small number in whom only one urinalysis was performed. The prevalence of renal dysfunction in healthy adults from the present cohort is therefore likely to be very low.

Plasma Cr values in children and adolescents were on the whole much lower than would be expected in an age-matched population from a developed country. This is likely to reflect decreased lean body mass due to malnutrition, given the very high proportion of children and adolescents with weights below the universally accepted age-standardised reference range [366,377,378]. The median weight for age in these...
subjects was 1.8 SDs below the standardised median and only 5% of these subjects had a weight above the standardised median. All subjects = 18 years of age had a plasma Cr below the middle of the age-specific reference range. The Cockcroft-Gault equation should not be used in children [370] and those equations that have been validated for use in children [379,380] rely on anthropometric measures not determined in the present study. Their interpretation would still be subject to a number of the limitations described in the preceding paragraph. No children or adolescents from the present study had persistent proteinuria on dipstick testing, therefore the underlying prevalence of renal dysfunction in subjects of this age was also likely to be very low.

The similar values obtained for FENOx in the PNG study population compared to that of Genyem subjects and Darwin controls (Chapter 11.3.2) also suggests that renal dysfunction was uncommon (FENOx has been shown to decrease in the setting of renal failure [96]). Given this assessment, it was decided that correction of plasma NOx and urine NOx/Cr results for renal dysfunction prior to statistical analysis would not be required in these subjects.

12.4.2. Relationship between malaria parasitemia and nitric oxide production

The striking finding from the present study, in contrast to the Genyem study (Chapter 11), was that no relationship was found between NO production and malaria parasitemia either cross-sectionally or longitudinally in any age group. Intra-individual fluctuation in urine NOx/Cr, plasma NOx and NOS activity over time was minimal despite clearance of microscopic parasitemia by anti-malarial drug therapy and subsequent recurrence of parasitemia in many subjects. There are at least four possible explanations for these findings, which will be discussed below:

1. Results from either location may have been due to chance or poor study design.
2. NO production may be saturated by non-malaria stimuli in PNG but not Papua province.
3. Parasites and/or humans are intrinsically different in the two locations.
4. Sub-microscopic levels of malaria parasitemia were more frequent in PNG and induce as much NO as higher levels.

12.4.2.1. Chance/poor study design?

Although the sample size in Genyem was smaller than in PNG (40 versus 195 included subjects), the Genyem subjects with and without malaria parasitemia were well matched in terms of age, sex, ethnicity and malaria exposure. Subjects with recent illness or medication use that may have influenced NO production were rigorously excluded from both studies. A maximum of 6 subjects were observed on the wards of the Genyem health centre by a member of the study team per night, so it is unlikely that breaches to the dietary fasting protocol occurred that may have influenced the results. The statistical differences found in Genyem were similar for both urine NOx/Cr and plasma NOx and withstood the loss of statistical power inherent in non-parametric tests. Likewise, different methods of classifying parasitemia based on the results of two expert microscopists made little difference to the statistical analyses. The likelihood that the Genyem results were due to chance was therefore probably less than 5%.

Two strengths of the PNG study were the larger sample size and the longitudinal design (above). Covariates such as age and sex were properly controlled for and parasitemia was defined by agreement of two very experienced microscopists following a rigorous cross-checking procedure. There were no differences in NO production attributable to the presence or level of malaria parasitemia at baseline, despite the larger sample size, and the differences in repeated measures from the same subjects longitudinally were very low regardless of the presence or absence of parasitemia. The stability of NO responses also suggests that deviation from the fasting protocol was either systematic, trivial or inconsequential. The possibility that both the cross-sectional and longitudinal observations were inaccurate or due to chance would therefore seem to be remote.
12.4.2.2. Saturation of nitric oxide production in Papua New Guinea but not Genyem?

It is possible that a level of stimulation sufficient to induce maximal or near-maximal NO production was achieved in most, if not all PNG subjects by factors other than malaria (as discussed in Chapter 11.4.3) but not in Papua Province. If this were so, then any additional effect of malaria parasitemia may not be evident in PNG. In support of this hypothesis is the observation that the GM plasma NOx for parasitic Genyem subjects was 36 µmol/L and for PNG subjects = 16 years was 34 µmol/L. The GM plasma NOx in aparasitemic Genyem subjects was 24 µmol/L whereas parasitemia was not associated with a difference in plasma NOx in PNG. These results were reflected in the respective GM urine NOx/Cr results in subjects = 16 years from Genyem and PNG, which were 0.15 and 0.16 in parasitic subjects, and 0.08 in aparasitic Genyem subjects. If the peak values are indicative of maximal NO production, then the higher baseline values in aparasitic PNG subjects may indicate that background stimulation from other factors is higher than in Genyem. An increment in NO production attributable to malaria may therefore not be seen if a ceiling was in place.

The two populations share a similar lifestyle and are likely to be subject to a similar spectrum of non-malarial infections (e.g., intestinal parasites) but it is impossible to gauge from the available literature whether levels of non-malarial parasite endemicity vary given the paucity of recently available data from both regions. Previous coprological surveys in Madang have shown universal infection with *Necator americanus* [381] and high rates of infection with other intestinal parasites have been described throughout PNG [382]. Intestinal parasitosis with helminths has been significantly associated with systemic NOx in one study [279], which further supports this hypothesis. This hypothesis could be addressed by correlating NO production with parasitemia before and after treatment of intestinal parasites (e.g., concurrent with periodic anti-helminth treatment, which is given routinely in some countries).
12.4.2.3. Intrinsic host and/or parasite differences?

There is very little data that supports or refutes whether intrinsic differences between malaria parasites exist that could potentially alter parasite virulence via differential induction of non-specific soluble mediators such as cytokines and NO. Strain-related variation in the ability of *P. falciparum* parasites to induce TNF-α production was found to be influenced by *Mycoplasma* contamination in a number of instances [119,120]. Interaction between different species of malaria parasites is also unlikely to be an explanation for the differences presented in the two studies here given that all four species occur in similar proportions in both locales [193,321] and species representation was not materially different in the two present studies. Genetic diversity between humans is well recognised though and it is quite plausible that intrinsic differences exist in any number or combination of genes that could influence NO production between subjects in Genyem and PNG. Genetic variation in geographically close populations like these has previously been demonstrated in other settings, for example with human leucocyte antigen (HLA) genotypes in East Africans [287]. Such differences may arise through: the founder effect (i.e., primarily determined by a common ancestor); migration in and out of an area; intermarriage and selective pressure exerted by various infectious diseases that may vary from location to location and within a particular location over time. Genetic polymorphisms that may influence NO production are the subject of Chapter 13.

12.4.2.4. Low-threshold induction of nitric oxide by sub-microscopic parasitemia?

Sub-microscopic but PCR-detectable levels of *P. falciparum* parasitemia have been shown to induce high levels of PBMC NOS activity in non-immune human volunteers from Brisbane given ultra-low doses of trophozoites in a proof-of-concept vaccine trial [383]. Although the kinetics of acute NO production in previously naïve volunteers could differ from those involved in chronically stimulated production, this may suggest that NO production in relation to malaria infection is an “all or none” phenomenon. Given the very high intensity of malaria transmission in the villages participating in the PNG study relative to nearby villages [17], it is plausible that a majority of those without parasites present on their baseline blood smear had infection below the microscopic level of detection.
PCR-only detectable infection appears to be common in village children from the Madang region 50 km south of the present study site [220] and widespread sub-microscopic infection has been demonstrated by PCR in adults from the Sepik region of PNG [21]. Only a small subset of randomly selected aparasitemic subjects had PCR performed on blood spots from T₀, of which 25% were positive for P. falciparum and 3% for P. vivax. Whether sub-microscopic parasitemia is any more common in Madang than in Genyem is debatable though, especially given that a higher proportion of screened subjects = 16 years in Genyem were microscopically parasitemic than Madang subjects. More frequent use of antimalarial medication in Genyem prior to the study may have resulted in more subjects having cleared parasitemia than in PNG but this seem unlikely given that chloroquine was the only readily available medication in Papua province (personal observation).

It is also possible that very widespread resistance to the drugs administered to the PNG subjects resulted in persistence of parasitemia below the level of detection by microscopy and the PCR used in the present study. There have been few published reports describing rates of parasite resistance to sulfadoxine-pyrimethamine in PNG but recent evidence is similar to a previous report [384] in suggesting that the rate of double mutations in the dihydrofolate reductase (DHFR) gene of P. falciparum isolates from the Madang region is very low and that triple and quadruple DHFR mutations and dihydropteroate synthase mutations are rarely being seen (Ged Casey, personal communication). This pattern and frequency of mutations is not likely to be associated with high rates of parasitological or clinical failure [385] and might suggest that those failures observed in the present study were more likely to be due to failure to take the study medications than to parasite resistance. P. vivax resistant to chloroquine has been reported in PNG and appears to be more widespread [386]. In the absence of directly-observed radical curative therapy, it is impossible to be certain whether extremely low level parasitemias may have induced equivalent levels of NO production as microscopically detectable parasitemia in the present study. Such therapy may be difficult to justify in a future longitudinal study because of the higher side effect profile and also emerging concerns that the occurrence and/or severity of malaria attacks following clearance of asymptomatic parasitemia may be
increased [387,388]. This is further discussed in relation to declining anti-toxic antibody levels following treatment in Chapter 14.4.

12.4.3. Age-related production of nitric oxide

It had been hypothesized prior to the present study that NO production would be highest in early childhood and decline into adulthood in a population exposed to intense malaria transmission [267]. This expected pattern of NO production would have mirrored the fever threshold curve describing the level of parasitemia required to elicit symptoms in different age groups [25,190] and would have supported the view that NO is a major mediator of malarial tolerance [179,267]. A consistent age-related pattern was not observed for the 3 indices of NO production measured in this study. It is difficult to discern the true relationship between urine NOx excretion and age in this sample due to possible distortion of the urine NOx/Cr ratio by age-related increasing Cr excretion [371] and the inherent inaccuracy of estimating NOx excretion from standardized values calculated in a different reference population [369]. Furthermore, just as the number of cells responsible for Cr production increase with age, so too would the number of NO producing cells, but it is impossible to know whether or not they do so in the same proportion. Estimated 24 hour NOx excretion and urine NOx/Cr were very highly correlated in adult subjects (whose Cr excretion is likely to change little with advancing age) which suggests the former may be a reliable indicator of NOx excretion at all ages. These measures were less well correlated in younger subjects, which probably reflects distortion in urine NOx/Cr values due to changing NOx and Cr excretion with age. The increase in NO producing cells with age almost certainly explains the apparent increase in 24 hour NOx excretion from a low in the 1-4 year age group to a peak in late adolescence. As in Genyem, urine NOx/Cr and NOS activity were poorly correlated (see discussion in Chapter 11.4).

In contrast, the best straight line fitting the relationship between plasma NOx and age was almost horizontal (i.e., consistent levels of plasma NOx across the age spectrum). Although there was significant variation in plasma NOx between age groups, the relationship did not conform to a recognisable pattern and none of the pairwise comparisons between different groups would have withstood statistical
correction for multiple comparisons. The statistically significant linear relationship between plasma NOx and age was “bolstered by the numbers” and otherwise of little biological significance. The different pattern of plasma NOx compared to estimated urine NOx excretion did not appear to be explained by fluctuation of FENOx with age as this variable was relatively stable across different age groups, and indeed, across a number of different populations. Plasma NOx may be a more sensitive indicator of recent NO production than urine NOx excretion, which conversely has been shown to be more stable over time [373].

Partial equalisation of inter-subject variation in plasma NOx could conceivably be related to circadian fluctuation of NO production that flattens out in the early morning. While this is highly speculative, circadian variation in NO production has been demonstrated in healthy human volunteers with plasma levels lowest just before 6 am [389] and nadirs in plasma NOx in healthy rats have been demonstrated to occur at 7.30 am [390] and 9 hrs after lights off [391]. It is important to note that dietary control may not have been utilised in the human study [389] and that other studies in humans have shown either no circadian variation [392] or different results to those previously cited [393].

What is abundantly clear from the examination of NO production in relation to age though is that none of the indices measured conformed to the hypothesis that motivated the present study. It is difficult to understand why adults would tolerate lower parasitemias than young children if NO were the major mediator of malarial tolerance, given that NO production was similar in adults and young children. The assumption that adults regulate malaria parasitemia at lower densities than children through more effective anti-parasitic immunity and that this triggers a proportionate tolerance-mediating response still seems reasonable, but the data from the present study suggest that the final mediator of this process is not NO. Another soluble mediator may fill this role and conform to the expected pattern (e.g., PGs). NO may be an intermediary or by-product of that pathway and it is possible that age-related mechanisms exist which govern differential production of the unknown mediator and NO. In that case, chronic elevated NO production may lead to other protective or pathological responses, or might even be redundant. Therefore, the present data do not exclude other roles for NO in relation to malaria infection.
12.4.4. Regulation of nitric oxide production

12.4.4.1. Immunoglobulin-E

It is likely that the potential stimuli of NO production in PNG would be similar to those in Genyem (discussed in Chapter 11.4.3). The present study has identified a possible link between chronic infection with intestinal helminths, IgE levels and PBMC NOS2 activity that is supportive of other recent evidence [278]. IgE levels were markedly elevated across the board compared to standard Australian reference values but still followed the usual pattern of increase from early childhood to the mid teens, followed by a slight decrease into late adolescence and adulthood. IgE levels (grouped into quintiles) significantly explained 4% of the overall variance in NOS activity. This is consistent with IgE contributing to overall NO production through induction of NOS2 via activation of PBMC CD23 receptors [89] but also suggests that other stimuli are more important for PBMC NOS induction.

12.4.4.2. C-reactive protein

CRP levels were measured as a general marker of the overall level of inflammatory responses [394] and were found to be modestly elevated in a small proportion of parasitemic subjects only. This is consistent with previous studies that have demonstrated high levels of CRP in subjects with clinical malaria [395] that correlated with severity [269], and levels of similar magnitude to the present study in asymptomatic parasitemic subjects [395,396]. CRP levels have been reported to correlate with the density of \textit{P. falciparum} parasitemia in asymptomatic Tanzanian children [397,398] and have been shown to decrease but remain elevated for at least 5 days following treatment for clinical malaria, and then normalise by 28 days [269].

CRP was significantly correlated with density of parasitemia in the present study but it is not possible to say whether recently resolved clinical malaria may have also contributed to raised CRP, as subjects were only asked about malaria symptoms in the preceding week. The correlation with parasite density extended below the standard cut-off that defines normal from elevated CRP, which is consistent with the
notion that low levels of inflammation can be associated with differences in CRP within the normal range [399]. Whether CRP has a role beyond indicating the degree of inflammation induced by malaria has received limited attention, however, CRP is thought to mediate a number of immunomodulatory responses [394], including anti-inflammatory responses in mice [400] and direct anti-parasitic effects against pre-erythrocytic stages of malaria [401,402].

More difficult to explain is the reason that plasma NOx was significantly reduced in subjects whose CRP was elevated as both CRP and NO are induced by a similar range of inflammatory cytokines, including IL-1, IL-6 and TNF-a [403]. One explanation for this finding could be that stable asymptomatic \textit{P. falciparum} infection induces production of a soluble factor that induces CRP but down-regulates NO, although the possible identity of such a candidate molecule is unclear and no association was found between parasitemia and NO production. Alternatively, if CRP was elevated but decreasing in this setting as a result of resolving clinical infection [397], then a cytokine associated with resolution of clinical malaria may have down-regulated NO production.

Sustained production of TGF-β has been postulated to control the transition between acute and resolving phases of malaria infection [404], by switching the cytokine response from Th1 to Th2-dominated in an apparent attempt to limit inflammation-associated pathology [405]. TGF-β may down-regulate CRP production [406] and is also a potent suppressor of \textit{NOS2} [407]. CRP itself, or another of the acute phase reactants induced in parallel with CRP, may have directly down-regulated NO production, although the present literature suggests that human CRP may induce rather than suppress \textit{NOS2} [408]. It is also possible that subjects with a genetically-determined capability for higher basal level NO production can more effectively down-regulate the Th-1 cytokines that induce CRP production [409] and vice versa, such that low level basal NO production leads to an enhanced inflammatory response to malaria.
12.4.4.3. The problem of “kus”

Epidemic viral upper respiratory tract infections (“kus” in Pidgin) affected the study population, especially at the follow up visits, but did not appear to affect NO production. This area has previously received limited research attention, with one study showing increased NO in exhaled air from normal subjects with a viral upper respiratory tract infection [410] and another showing no change in similarly measured nasal NO [411].

12.4.5. Conclusion

This study has shown that systemic and PBMC production of NO by healthy coastal Papua New Guineans is much higher than that of Darwin adults and shows a remarkably high level of stability over time regardless of malaria status or malaria treatment. Although this study is not definitive in ruling out malaria parasitemia as a major stimulant of NO production in this population, for this to be the case all of the following would need to be true: the level of sub-microscopic parasitemia would need to be extraordinarily high, and higher than could be detected by the PCR used in this study; the drug failure rate would need to be very high; and the threshold for NO induction would need to be extremely low (i.e., below the level of PCR detection in this study). For the reasons discussed above, it is extremely unlikely that all of these conditions were met, and it is far more likely that the alternative explanation is correct - that malaria parasitemia is not required to induce the high levels of NO production seen in PNG compared to Darwin.

The second major finding of this study was that NO production did not vary with age in any easily recognisable or biologically meaningful way. If the phenomenon of an age-dependent pyrogenic threshold truly exists, then the data from the present study argue that NO does not facilitate this, regardless of the reasons for its elevated production in this setting. A growing body of evidence suggests that the genetically determined capacity to produce NO may be important in determining the outcome of clinical malaria in endemic regions in which malaria and other infections might contribute to selection of polymorphisms. Understanding the complex regulation of NO in malaria-endemic environments is therefore important. Other than examining its role in tolerance of malarial parasitemia, this study was not designed to
investigate what other benefits (or disadvantages) elevated NO production might have in this population. However, the results from the present study, and the one in Genyem (Chapter 11), are generally supportive of the Thai studies [347] in tying together malaria, NO, helminth infections and IgE and suggest this is an area amenable to further research.

Concerns have recently been expressed regarding an increased risk of future clinical malaria following radical treatment of asymptomatic malaria parasitemia in semi-immune individuals. The proportion of subjects reporting recent symptoms of clinical malaria at each time-point in the current study was similar, despite parasitemia recurring in 47% of subjects between T₁ and T₂. Although this is reassuring, the current study was not specifically designed to address these concerns and future studies should be designed to either avoid treatment of asymptomatic individuals or follow up subjects to detect and treat clinical malaria (e.g., in vaccine trials).
Chapter 13. Genetic determinants of NO production in malaria-endemic areas: Polymorphisms in the genes encoding the interleukin-12 p40 subunit (IL12B) and NOS2 (NOS2) and their association with nitric oxide production and disease severity

Variation between individuals in NO production may in part be explained by different types and levels of stimuli, but the striking stability in NO production within individuals over time demonstrated in Chapter 12 could also be interpreted as suggesting that genetic determinants played a role. This is especially likely when the proposed genes of importance are associated with potential survival advantages that may have favored their selection. Both IL-12 and NO have been associated with malarial disease outcome, with levels inversely proportional to disease severity [96,290]. This chapter first explores whether novel polymorphisms in IL-12 are associated with malarial disease severity and NO production by examining these relationships in an established Tanzanian database of children with and without severe disease. The same polymorphisms, as well as all of the previously determined NOS2 gene polymorphisms of purported significance, were then examined in relation to IL-12 and NO production in the PNG cohort.

13.1. General introduction

Case-control studies investigating the association between genetic polymorphisms and disease outcomes are a powerful and efficient means of identifying the role that innately determined effector mechanisms play in susceptibility or resistance to disease. This approach “cuts to the chase” because it can potentially identify critical determinants that are operative in the context of the human situation irrespective of what may be suggested by in vitro and animal studies. An array of resistance and susceptibility alleles have been discovered in relation to malaria that vary markedly in their distribution, both within continents and across the globe (reviewed in [412]). This regional variation, along with the fact that a number of susceptibility alleles have been maintained at moderate intensity, highlights the nuances involved in selective pressure at the population level. It has been suggested that variation in transmission intensity and parasite-determined virulence factors may underlie some
of the regional variation and that the frequency of mutations may reach equilibrium if they are deleterious in some respects but beneficial in others (so-called “balanced polymorphisms”) [287].

The occurrence and prevalence of disease-associated polymorphisms may also vary geographically depending on whether the polymorphism itself is the critical determinant of altered gene activity or is a marker of linkage to a “true” or critical polymorphism in the same or nearby gene. This non-random linkage may vary from a very “loose” association to a very “tight” association depending on the distance between the two genetic elements, and is referred to as linkage disequilibrium. Thus, a polymorphism with apparent geographically restricted relevance may still be indicative of a universally important functional association if in linkage disequilibrium with a critical genetic determinant. Alternatively, apparently significant associations may be due to chance, the likelihood of which may be expanded quite markedly if multiple potential associations are considered without any statistical adjustment for the number of comparisons. Collectively, this highlights the need for subjecting apparent genetic associations to examination in different populations and also the importance of follow-up studies aimed at correlating the polymorphism with its supposed functional outcome. If functional associations differ, then this should prompt investigators to search for related factors on the same gene, nearby genes and genes that control expression of inter-related molecules to dissect the complex pathological processes involved. Such research should be informed by the context of local factors relating to differences in malaria epidemiology and disease phenotypes.

The following studies were founded on the premise that genetic polymorphisms under selection pressure from malaria are most likely to influence susceptibility to death from severe malaria prior to the onset of effective exposure-related immunity. In the first study (Chapter 13.2), two polymorphisms from different regions of a gene controlling IL-12 expression were examined in relation to outcome from malaria and production of NO in a previously characterised population of Tanzanian children [96]. One of these polymorphisms had previously been associated with susceptibility to type 1 diabetes (T1D) in British and Australian families [413] and the other was a novel polymorphism found by extending previous studies along the IL12B gene
The second study (Chapter 13.3) explored the frequency of polymorphisms in both the *IL12B* and *NOS2* genes in the PNG cohort (Chapter 12.2.3), and examined their effect on basal NO production in a setting not confounded by the exuberant cytokine responses evident with acute disease.

### 13.2. Polymorphisms in the *IL12B* gene and their association with mortality from cerebral malaria and NO production in Tanzanian children

#### 13.2.1. Introduction

Interleukin-12 is a heterodimer composed of a p40 subunit (encoded by the *IL12B* gene) and a p35 subunit. A polymorphism associated with susceptibility to development of T1D has been described in the 3’ untranslated region of the *IL12B* gene and is referred to as *IL12B*-3’UTR allele-1 (Chapter 9.6; [413]). This polymorphism was also associated with higher levels of basal IL-12 expression *in vitro* by Epstein Barr virus-transformed cells that were homozygous for *IL12B*-3’UTR allele-1 compared to *IL12B*-3’UTR allele-2. The *IL12B*-pro polymorphism is a complex insertion-deletion polymorphism that resides in the promoter region upstream of the *IL12B*-3’UTR polymorphism and results in 2 alleles that differ in size by 4-bp (Chapter 9.6). This polymorphism was defined just prior to the present study so nothing was known of its disease or functional associations.

On the basis of animal studies and clinical studies (reviewed by [414]), IL-12 has been postulated to mediate protective responses in human malaria, possibly through its ability to promote IFN-? production and/or subsequent stimulation of NO [415]. It is unclear to what degree the proposed protective effects of IL-12 are modified by an innate ability of the host to control IL-12 expression and/or are subject to regulation by other cytokines. For example, a study in Gabon showed decreased IL-12 responses in children with severe malaria relative to mild malaria [290] which would be consistent with genetic susceptibility to severe disease in children less capable of mounting an IL-12 response. However, levels of down-regulatory cytokines were also increased in the children with severe disease and basal production of IL-12 recovered to equivalent levels in survivors of mild and severe disease, which argues against a genetic deficiency in IL-12 production.
The purpose of the present study was therefore to explore associations between the two polymorphisms in *IL12B*, severity of malaria and mortality from severe malaria in children with uncomplicated or cerebral malaria compared to healthy controls. The possible functional association of each polymorphism with production of NO, a downstream mediator of IL-12 activity and a proposed mediator of protection against severe malaria, was examined by investigating the effect of genotype on urine NOx/Cr and plasma NOx/Cr ratios. Given the previous findings in T1D, it was hypothesised *a priori* that *IL12B*-3’UTR allele-2 homozygosity would be associated with lower levels of IL-12 production and therefore an increased risk of developing and dying from cerebral malaria. As it was unclear which *IL12B*-pro allele might be associated with increased IL-12 expression, the association between *IL12B*-pro genotype and malaria severity was examined initially and the relationship with NO production examined in light of hypotheses derived from the disease association study.

13.2.2. Methods

13.2.2.1. Study site

Subjects were recruited from a hospital population in the coastal city of Dar es Salaam, the former capital of Tanzania, which is located in east Africa between similar latitudes to the island of New Guinea (Chapter 12.2.1). The country’s population structure and mortality rates at the time of the study were typical of developing countries and it’s GDP per capita ranked amongst the poorest countries of the world [416]. Malaria is prevalent in over 80% of habited areas in Tanzania and is a major cause of morbidity and mortality, especially in children < 5 years of age [417]. The vast majority of malaria cases (greater than 90%) are due to *P. falciparum*, with the remainder caused by *P. ovale* and *P. malariae* [417].

The clinical epidemiology of malaria in coastal regions such as Dar es Salaam, where peaks of transmission and morbidity follow the onset of the rainy seasons in March-June and November-January, is markedly different to areas of the country with much higher transmission such as Ifakara and the region around Lake Victoria. Parasite
prevalence rates in Dar es Salaam schoolchildren ranged from 3% to 46% in a 1994 malarialmetric survey [417], whereas perennial rates in excess of 80% are common in inland holoendemic regions [418]. Cerebral malaria presents later in urban children, with a peak incidence around 3-4 years of age [282], whereas cerebral malaria in regions of higher transmission is comparatively less common [419] and is more likely to occur in infants and younger children.

13.2.2.2. Subjects

Blood and urine samples were obtained from a cohort of 191 children aged between 6 months and 9 years who were admitted to the Muhimbili Medical Centre in Dar es Salaam between May 1994 and January 1995. For the purposes of the present study, children with WHO-defined clinical malaria caused by *P. falciparum* have been classified as having either cerebral malaria with unrousable coma (CM; n=86) or uncomplicated *P. falciparum* malaria (UM; n=55). In addition, 50 healthy control (HC) children were recruited from pediatric and surgical wards who had no fever history within the past 2 weeks; a normal white cell count and no acute illness. Ten of the HC children had asymptomatic malaria parasitemia on blood smear (GM parasitemia 595 trophozoites/µL) and the rest were aaparasitemic. PCR amplification of 2 different *P. falciparum* gene fragments was performed on 35 of the 40 aparasitemic subjects, of which 13 were positive [282].

13.2.2.3. Genotyping for *IL12B* polymorphisms and measurement of urine NOx

DNA was amplified from blood filter spots by whole genome amplification (Chapter 9.5) and then genotyped for the *IL12B*-3′UTR and *IL12B*-pro polymorphisms as previously described (Chapter 9.6). Samples were unavailable for genotyping, or genotyping failed, in 13 of the 191 children from the original cohort.

Urine and plasma NOx were measured after an overnight fast in the HC children. It is likely that the dietary ingestion of nitrate in urban Tanzanian children would be low given that few of the commonly eaten foods are high in nitrates [96]. As it was not possible to fast children who were clinically unwell with malaria, the time since ingestion of last food and drink was documented in the UM and CM children at the
time of recruitment. In addition, recent dietary histories were taken from the HC children. Urine NOx was measured using *Pseudomonas oleovorans* nitrate reductase coupled with the Griess reaction [420], the general principles of which are similar to the procedure described in Chapter 9.2. As with the Genyem and Madang field studies, urine NOx was expressed as a ratio of urine Cr to account for differences in urinary concentration between subjects. Additionally, plasma NOx was expressed as the ratio of plasma NOx to plasma Cr to account for the differences in renal function that were observed between the groups.

### 13.2.2.4. Statistical analyses

Statistical analyses were generally performed, and are presented, as described in Chapter 10. Based on the *a priori* hypothesis that *IL12B*-3’UTR allele-2 homozygotes would show increased susceptibility to disease, subjects with this genotype were compared to all other subjects in the statistical models. *IL12B*-pro genotypes were initially considered separately but then segregated into *IL12B*-pro allele-1 homozygotes versus other genotypes based on analyses showing an increased risk of death from CM in this group (below). Predicted outcomes from CM were modelled using logistic regression, controlling for factors expected to influence outcome. Previously identified significant covariates were examined in these models, including disease severity (ordered HC, UM, CM), age (years), density of parasitemia (parasites/µL), and plasma Cr (mmol/L; for urine NOx/Cr analysis only) [96]. Deviation of genotypes from the expected frequencies under Hardy-Weinberg equilibrium was examined using likelihood-ratio testing [421].

### 13.2.3. Results

#### 13.2.3.1. Baseline characteristics

The baseline characteristics of the study group are given in Table 13.1. Of the 50 HC children, 29 (58%) were healthy children awaiting an elective surgical procedure and 21 were receiving bed rest for a fractured long bone of greater than 1 week duration. Only 3 (6%) of the 50 HC children gave a dietary history of recent ingestion of food potentially high in nitrate.

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Table 13.1. Baseline characteristics of Tanzanian children

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>UM</th>
<th>CM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>50</td>
<td>55</td>
<td>86</td>
</tr>
<tr>
<td>Mean age (yr)</td>
<td>4.3</td>
<td>3.1</td>
<td>3.5</td>
</tr>
<tr>
<td>Range (yr)</td>
<td>0.5-9.6</td>
<td>0.5-7.8</td>
<td>0.6-7.2</td>
</tr>
<tr>
<td>Mean weight (kg)</td>
<td>13.9</td>
<td>12.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Mean time since last food (hr)</td>
<td>13.0</td>
<td>5.6</td>
<td>16.8</td>
</tr>
<tr>
<td>Mean time since last fluid (hr)</td>
<td>12.2</td>
<td>2.6</td>
<td>13.8</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>64</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Geometric mean parasitemia (trophozoites/µL)</td>
<td>*585</td>
<td>45,624</td>
<td>32,640</td>
</tr>
</tbody>
</table>

*Parasitic subjects only. HC = Healthy control; UM = Uncomplicated malaria; CM = Cerebral malaria

13.2.3.2. Association between IL12B polymorphisms, clinical malaria and outcome from cerebral malaria

Genotyping data were generated for the 178 Tanzanian subjects from whom DNA samples were available (Table 13.2). The overall allele frequency for IL12B-pro allele-2 was 0.60 and for IL12B-3’UTR allele-1 was 0.59. IL12B-pro genotypes were in Hardy-Weinberg equilibrium (P=0.160), but IL12B-3’UTR genotypes were not, with heterozygotes being under-represented compared to both groups of homozygotes (P<0.001). Similar frequencies (0.40 for IL12B-pro allele-1 and 0.65 for IL12B-3’UTR allele-2) and distributions (P=0.751 and P=0.003 respectively) were observed in the healthy controls. In a chi-squared analysis and separate chi-squared analysis for trend, there was no significant difference in IL12B-pro or IL12B-3’UTR genotype between children who were asymptomatic and children with either uncomplicated or cerebral malaria. However, the IL12B-pro polymorphism was associated with a significantly different likelihood of death from CM in an unadjusted 3 by 2 chi-squared analysis (P=0.024). Individuals homozygous for IL12B-pro allele-1 were over-represented amongst the children who died from CM (Table 13.2). As there was no difference in the likelihood of death from CM between IL12B-pro heterozygotes and IL12B-pro allele-2 homozygotes, these
subjects were considered together as a single group. The unadjusted OR for death from CM in IL12B-pro allele-1 homozygotes was 5 (95% CI 1.4-18.2; P=0.013 (Table 13.2). The best model explaining the likelihood of death from CM was the one that controlled for IL12B-pro genotype and parasitemia as the only significant variables (for IL12B-pro allele-1 homozygotes, adjusted OR 7.0; 95% CI 1.7-28.5; P=0.007). Including the non-significant variables age and IL12B-3’UTR genotype did not materially alter the significance of this relationship (adjusted OR 5.6; 95% CI 1.1-27.6; P=0.035).

Table 13.2. Frequency of IL12B polymorphisms and genotypes within each disease category in Tanzanian children and odds ratio for death from CM

<table>
<thead>
<tr>
<th>Subjects tested</th>
<th>Total</th>
<th>IL12B-pro genotype</th>
<th>IL12B-3’UTR genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>11 12 or 22</td>
<td>11 or 12 22</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy currently</td>
<td>43</td>
<td>6 36</td>
<td>30 9</td>
</tr>
<tr>
<td>Uncomplicated malaria</td>
<td>53</td>
<td>6 47</td>
<td>40 9</td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td>12 83</td>
<td>70 18</td>
</tr>
<tr>
<td>Cerebral malaria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survival</td>
<td>59</td>
<td>5 54</td>
<td>47 12</td>
</tr>
<tr>
<td>Fatal outcome</td>
<td>23</td>
<td>7 15</td>
<td>13 9</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>12 69</td>
<td>60 21</td>
</tr>
<tr>
<td>CM survival (%)</td>
<td>72</td>
<td>42 78</td>
<td>78 57</td>
</tr>
<tr>
<td>CM death odds ratio¹</td>
<td>5.0</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>1.4-18.2</td>
<td>0.9-7.8</td>
<td></td>
</tr>
<tr>
<td>P value¹</td>
<td>0.013</td>
<td>0.065</td>
<td></td>
</tr>
</tbody>
</table>

Genotypes were determined for IL12B promoter and 3’UTR polymorphisms in Tanzanian children who were malaria-exposed but otherwise healthy at the time of study (HC); from children diagnosed with uncomplicated malaria (UM); and from children with cerebral malaria (CM). The latter were divided into subgroups based on survival of the CM episode. Data were unobtainable for the promoter
polymorphism in one HC subject, and for the *IL12B*-3′UTR in four subjects from each of the HC and UM groups. Disease outcome was not recovered for one CM subject who was thus excluded from survival analyses. Unadjusted odds ratios and P values are presented.

Considering the *IL12B*-3′UTR polymorphism, the crude OR for death from CM in allele-2 homozygotes was 2.7 (95% CI 0.9-7.8; P=0.067), which was not materially altered by adding parasitemia to the model (OR 3.0; 95% CI 0.9-9.7; P=0.065). When the promoter genotype was added to the multivariate model, the OR for *IL12B*-3′UTR allele-2 homozygosity was reduced to 1.5 (95% CI 0.4-6.6; P=0.564) suggesting that the nearly significant *IL12B*-3′UTR effect may be explained at least in part by linkage disequilibrium with *IL12B*-pro allele-1.

Haplotype was significantly associated with death from CM in an unadjusted proportionally fitted log-linear model (P=0.031), with the highest risk attributable to the *IL12B*-pro allele-1/3′UTR allele-2 haplotype. The OR for death attributable to this haplotype (which was estimated to occur at a frequency of 30% overall in subjects with CM compared to all other haplotypes) was 2.7 (95% CI 1.2-5.9; P=0.007). Seven (78%) of 9 subjects known to be homozygous for this haplotype died from CM, compared to 15 (21%) of the 72 remaining subjects (some of whom may also have been homozygous for the allele-1/2 haplotype). After controlling for parasitemia, the OR for death in subjects known to be allele-1/2 homozygous was 16.1 (95% CI 2.8-93.8; P=0.002).

**13.2.3.3. Association between *IL12B* polymorphisms and nitric oxide production**

Both urine NOx/Cr and plasma NOx/Cr have previously been shown to be significantly inversely associated with disease severity in these children, with levels lowest in children with CM [96]. In a univariate analysis, the GM urine NOx/Cr ratio was 0.11 (95% CI 0.07-0.15) in *IL12B*-pro allele-1 homozygotes compared to 0.16 (95% CI 0.14-0.19) in all other subjects (P=0.027). *IL12B*-pro allele-1 homozygosity was independently predictive of lower NOx excretion after controlling for disease severity F=0.012; Figure 13.1). The significant predictive effect of *IL12B*-pro genotype was maintained after controlling for the covariates age, plasma...
Cr, parasitemia and *IL12B*-3’UTR genotype (P=0.038). Mean urine NOx/Cr was lowest in *IL12B*-3’UTR-2 homozygotes in each disease category, (Figure 13.2) but this was not statistically significant, either in a univariate model (P=0.066) or after controlling for disease severity (P=0.077).

![Malaria Status](image)

**Figure 13.1.** Association between *IL12B* promoter polymorphism and urine NOx/Cr in Tanzanian children

Disease categories: Healthy controls (HC), uncomplicated malaria (UM), cerebral malaria (CM). Unshaded bars *IL12B*-pro allele-1/1 and shaded bars (all other *IL12B*-pro genotypes) represent the mean of each group and whiskers, the 95% confidence intervals.
Figure 13.2. Association between $IL12B$ 3’UTR polymorphism and urine NOx/Cr in Tanzanian children

Disease categories: Healthy controls (HC), uncomplicated malaria (UM), cerebral malaria (CM). Unshaded bars ($IL12B$-3’UTR allele-2/2) and shaded bars (all other $IL12B$-3’UTR genotypes) represent the mean of each group and whiskers, the 95% confidence intervals.

The association between plasma NOx/Cr and $IL12B$ genotypes was investigated as above. In a univariate analysis, the GM plasma NOx/Cr ratio was 0.52 (95% CI 0.40-0.68) in $IL12B$-pro allele-1 homozygotes compared to 0.76 (95% CI 0.69-0.84) in all other subjects (P=0.004). $IL12B$-pro allele-1 homozygosity was independently predictive of lower plasma NOx/Cr after controlling for disease severity (P=0.002). The significant predictive effect of $IL12B$-pro genotype was maintained after controlling for age, parasitemia and $IL12B$-3’UTR genotype (P=0.002). Mean plasma NOx/Cr was similar in $IL12B$-3’UTR homozygotes and subjects with other genotypes both overall (P=0.335) and in each disease category. Likewise, there was no significant association between homozygosity for the combined $IL12B$-pro allele-1/3’UTR allele-2 and either measure of NO production.
Subjects whose age was amongst the lowest 50% in the sample (\(\leq 3.7\) years) and who died from CM had a significantly lower urine NOx/Cr than those who survived (P=0.010). However, this difference was not observed in older subjects and may have been explained by the significantly higher plasma Crs (P=0.005) or parasitemias (P=0.006) in the children who died compared to survivors. Plasma NOx/Cr was lower in the younger half of children with CM who died compared to survivors but this difference was not significant (P=0.141). Likewise, there was no difference in plasma NOx/Cr between the two groups of older children.

13.2.4. Discussion

This study was the first to define both a disease and a functional association for a newly described polymorphism in the promoter region of the gene that encodes the IL-12 p40 subunit, \textit{IL12B}. Children homozygous for \textit{IL12B}-pro allele-1 were approximately seven times more likely than children with other genotypes to die from CM once it had developed, but this genotype was no more common in children with uncomplicated or CM than in asymptomatic controls. The near-significant increased risk of death from CM in children homozygous for the previously described \textit{IL12B}-3’UTR allele-2 was likely to be mostly explained by linkage disequilibrium with \textit{IL12B}-pro allele-1. However, the \textit{IL12B}-pro allele-1/3’UTR allele-2 haplotype was independently associated with a higher risk of death relative to all other haplotypes, which may suggest both polymorphisms are important or alternatively in linkage disequilibrium with a more critical polymorphism. Individuals homozygous for \textit{IL12B}-pro allele-1 also had significantly lower urine NOx/Cr and plasma NOx/Cr ratios than those with other \textit{IL12B}-pro genotypes both in the crude analysis and after adjusting for the previously described effect of disease severity [96].

These results suggest that genetically determined differential expression of IL-12 by Tanzanian children does not alter the likelihood of developing uncomplicated or CM but does strongly influence the outcome of CM once it has occurred. This apparent paradox is consistent with the findings of previous studies and is amenable to explanation. IL-12 production was found to be decreased in Gabonese children with
severe (non-cerebral and non-fatal) malaria relative to HCs and children with UM [290] but basal production of IL-12 was similar in the different groups after recovery [97,290]. The reduction in IL-12 observed in children with severe malaria may have resulted from the up-regulation of IL-10 in response to high levels of TNF-a rather than genetic variation between the subjects [291]. The additional administration of low-dose IL-12 concurrent with a sub-curative dose of chloroquine to rodents with established \textit{P. chabaudi} AS malaria resulted in 100% survival compared to 30% mortality in mice treated with chloroquine alone [422]. This effect was unlikely to be dependent on the choice of antimalarial as similar results were observed using clindamycin in place of chloroquine [414]. Collectively these studies are consistent with the notion that differences between individuals in IL-12 expression are most important in the setting of severe disease, whereby even small increments in IL-12 production may critically influence outcome when combined with treatment.

Possession of at least one \textit{IL12B}-pro allele-2 by subjects in the present study was associated with evidence of increased NO production in healthy children and diseased children, however, it is not clear whether NO mediated the protective effect against death from CM. Although urine NOx/Cr was significantly lower in children who died compared to survivors of CM in the youngest 50\textsuperscript{th} percentile, this difference was not seen in older children or with plasma NOx/Cr and may have been confounded by renal failure and parasitemia. It is plausible though that the disease and functional associations demonstrated in the present study were linked. In the aforementioned rodent study [422], the effect of IL-12 administration was associated with increased IFN-\gamma production, which appears to be essential as the protective effect of IL-12 is lost in IFN-\gamma gene deficient mice [414,423]. Pre-treatment of mice and monkeys with IL-12 resulted in complete protection against sporozoite challenge with \textit{P. yoelii} [294] and \textit{P. cynomogli} [203] respectively, which was shown to be partly dependent on induction of IFN-\gamma and subsequent stimulation of NO. Protection against blood-stage malaria caused by \textit{P. chabaudi} AS has also been attributed to NO by some authors [415] but discounted by others using \textit{NOS2} knockout mice [424]. Therefore, while it is possible that the protective effect of IL-12 in children with CM was mediated by NO, the equivocal results from other studies demand that this functional association be subject to further scrutiny.
The same two $IL12B$ polymorphisms were examined in a Kenyan population of 693 children with severe malaria [425] in which both were found to be in Hardy-Weinberg equilibrium, but no significant association was found between either genotype and mortality from severe malaria [426]. Similarly, there was no association between these $IL12B$ genotypes nor the $IL12B$-pro allele-1/3’UTR allele-2 haplotype and outcome in the subgroups with cerebral and non-cerebral severe malaria. The divergent results from Tanzanian and Kenyan children are typical of a number of studies examining the effect of genetic polymorphisms on susceptibility and outcome from malaria in different African populations [287] but may also have been due to chance. Although geographically close, genetic heterogeneity in HLA types has been demonstrated previously between Kenyans and Tanzanians [427]. As an example of how a recessive HLA-linked gene might interact with $IL12B$: the association that was found between $IL12B$-3’UTR allele-1 and susceptibility to T1D was evident in HLA-identical but not in HLA-mismatched sibling pairs [413].

It is also possible, and probably likely given the present results, that the $IL12B$ polymorphisms are in linkage disequilibrium with one or more polymorphisms that influence gene expression and downstream NO production in either the Tanzanian children or the Kenyan cohort. Unfortunately, it was not possible to measure indices of NO production in the Kenyan cohort to examine the relationship with the $IL12B$ polymorphisms. Another possibility is that genetic polymorphisms in the genes that regulate expression of other mediators that may interact with IL-12 (such as IL-10, TNF-a or even NO) are present at different frequencies in the two cohorts. Finally, differences in the intensity of malaria transmission between the two locations may be reflected by different rates of acquisition of anti-severe disease immunity. This can subsequently lead to differences in disease phenotype [11], which may have influenced the responsiveness of children to differential expression of IL-12.

While it is conceivable that either of the significant results in the Tanzanian study could have occurred by chance, the odds of chance accounting for two different and plausibly linked phenomena at the same time would be lower than just one of these occurrences. One of the more interesting findings was that the $IL12B$-pro allele-1/3’UTR allele-2 haplotype was associated with increased death from CM but known homozygosity for this haplotype was not associated with decreased NO production.
compared to all other subjects. This may suggest that linkage of the two alleles leads to increased mortality via a mechanism that relies on IL-12 but not on NO, and that only possession of \textit{IL12B}-pro allele-2 independent of \textit{IL12B}-3'UTR allele-1 is required to increase NO production. Another somewhat paradoxical finding to emerge was that the polymorphism strongly associated with death from CM in one East African population did not appear to have the same effect in a nearby population. Taken together, these observations highlight the fact that a multitude of pathophysiological processes interact during an episode of clinical malaria, some of which are tuned by previous exposure to parasites (which in themselves are polymorphic) and all of which are ultimately genetically regulated. Some of these are powerfully protective across different populations (such as the sickle cell trait) and others (that may include a critical \textit{IL12B} polymorphism) are undoubtedly more subtle and subject to modulation.

The results from the East African studies therefore emphasise the importance of combining genetic and functional analyses wherever possible, as well as providing information to inform the design of future studies that aim to further test the significance of the positive associations that have been described.

13.3. Polymorphisms in the \textit{IL12B} gene and \textit{NOS2} gene and their association with basal IL-12 and NO production in Papua New Guinean children and adults

13.3.1. Introduction

Three polymorphisms in the \textit{NOS2} promoter have also been associated with malarial disease outcome: a CCTTT microsatellite repeat \([(CCTTT)\textsubscript{n}]\) located \(~2.5\) kb upstream of the \textit{NOS2} transcription start site [284]; a single nucleotide substitution from G\rightarrow C at position -954 (G-954C) [283], and another from C\rightarrow T at position -1173 (C-1173T) [285]. Conflicting results have been obtained from studies of the \(\text{(CCTTT)}\textsubscript{n}\) polymorphism both \textit{in vivo} and \textit{in vitro}. Shorter forms of the CCTTT repeat (both alleles = 10 repeats) and a reduced summed repeat length were associated with fatal CM in Gambian children [284], whereas longer forms (either allele = 15 repeats) and increased summed repeat length were associated with severe malaria in Thai adults [286]. In contrast, CCTTT repeat length was not associated
with malarial disease severity or NO production in the Tanzanian children (Chapter 13.2.2.2; [317]) or Gabonese children [280], nor malarial disease severity in Kenyan children [285]. IL-1ß-induced NOS2 transcription in transfected colonic carcinoma cells was reported to be most effective in constructs with (CCTTT)$_{14}$ [428]. Another in vitro study reported no differences in basal or cytokine-induced NOS2 promoter activity by constructs with deletions in the CCTTT repeat region [429].

Reports on the functional relevance of the G-954C polymorphism have also led to differing conclusions. This polymorphism was over-represented in Gabonese children with mild compared to severe malaria [283] and was associated with an increased time to symptomatic reinfection [280], however, the G-954C mutation was not associated with malarial disease severity in Tanzanian children [317]. As geographic variation in 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 unclear. Likewise, a major question to arise from the Tanzanian and Kenyan studies (Chapter 13.2.4) was whether the functional association between the IL12B-pro polymorphism and NO production may be due to chance or to linkage disequilibrium with a critical polymorphism. Because of the importance of examining such polymorphisms in different geographic locations and correlating them with functional outcomes, and the conflicting results from these studies, the aforementioned polymorphisms in IL12B and the NOS2 promoter were examined in the cohort from PNG.

In this study, we hypothesised that basal IL-12 and NO production during good health in a population chronically exposed to high level malaria transmission would be positively correlated, in line with the general observation that IL-12 is a potent stimulator of NO expression [97] and the association shown in our previous study [426]. Further, we hypothesised that IL-12 and NO production would be increased in subjects with an IL12B-pro allele-2 and/or and IL12B-3’UTR allele 1, and that malaria parasitemia would be inversely correlated as shown in two previous studies [290,430]. Basal NO production was also tested in relation to the three NOS2 polymorphisms using hypotheses generated a priori from the previous studies [284,286,428].
13.3.2. Methods

For the *IL12B* study, subjects were the 195 children and adults enrolled in the PNG study who were not excluded at $T_0$ (Chapter 12.2.3). Genotyping was attempted for all 216 enrolled subjects in the *NOS2* studies. Genotyping of the *IL12B* and of the G-954C *NOS2* polymorphisms was performed as described in Chapter 9. The number of CCTTT repeats in the *NOS2* promoter ~2.5 kb 5’ to the transcription start site and the presence of the C-1173T polymorphism was determined by Maurine Hobbs (University of Utah and VA Medical Centers, US) in WGA DNA product (Chapter 9.5) as previously described (Chapter 9.8 and [285,317]). Urine NOx/Cr, estimated NOx excretion, plasma NOx and NOS activity were measured as described in Chapter 9. IL-12 was measured by Sujittra Chaisavaneeyakorn (Center for Disease Control, US) using an OptEIA kit (PharMingen, US) as previously described [431]. Data from $T_0$ only was used for the *IL12B* study, whereas additional data from $T_1$ was utilised from subjects who received no treatment at $T_0$ but in whom baseline urine or plasma was available only on the latter occasion for the *NOS2* study. Statistical analyses were generally performed and are presented as described in Chapter 10.

13.3.3. Results

13.3.3.1. Plasma IL-12 levels, parasitemia and nitric oxide production

Plasma IL-12 was measured by ELISA at $T_0$ in 161 subjects who were included in the statistical analysis (in addition to those subjects for whom no specimen was available [Chapter 12.3.1.4], technical problems with assay reagents meant that no result was available for 15 samples with consecutive identification numbers from the end of the dataset). The GM IL-12 in all subjects was 207 pg/mL (95% CI 172-248) and there was no significant relationship between IL-12 and sex, age in years or age-group. IL-12 levels were significantly inversely correlated with PBMC NOS activity in the whole cohort ($r= -0.22; P=0.007$). Each two-fold increase in NOS activity was predictive of a 25% (95% CI 7-38) decrease in plasma IL-12 levels. There was no significant relationship between IL-12 and plasma NOx ($P=0.99$) and neither was plasma NOx correlated with PBMC NOS activity.
There was no difference in IL-12 levels between subjects with any malaria parasitemia and those who were aparasitemic (P=0.23) nor between subjects whose only parasitemia was due to *P. falciparum* and aparasitemic subjects (P=0.35). However, the GM IL-12 was 31% (95% CI 1-52) lower in subjects with parasitemia including *P. falciparum* infection (175 pg/mL) than in the combined group of aparasitemic subjects and subjects with other parasite species (252 pg/mL; P=0.045). Furthermore, *P. falciparum* density and IL-12 were strongly but inversely correlated (r= -0.41; ?= -0.45; P<0.001 for both; Figure 13.3), with IL-12 predicted to decrease by 19% (95% CI 10-27) for each two-fold increase in *P. falciparum* parasitemia. The significance of the predictive effect of NOS activity on IL-12 levels was reduced (P=0.09) but that of *P. falciparum* density was not (P=0.001) when included together in the regression model. Controlling for the non-significant effect of age made no difference to the magnitude or significance of any of these relationships.
Figure 13.3. Relationship between plasma interleukin-12 and *P. falciparum* asexual parasite density in PNG subjects

Individual values are represented by circles. The straight line is the regression line showing a significant negative inverse relationship ($r^2=0.17; P<0.001$).

13.3.3.2. *IL12B* polymorphisms

Genotyping data for *IL12B*-pro and *IL12B*-3’UTR were available in all but 4 and 7 subjects respectively (due to failure of amplification) who were not excluded at T₀. The gene frequency for *IL12B*-pro allele-2 was 0.17 and for *IL12B*-3’UTR allele-1 was 0.38 (Table 13.3). *IL12B*-pro genotypes were in Hardy-Weinberg equilibrium (P=0.794) but *IL12B*-3’UTR genotypes were not (P=0.002), with heterozygotes being under-represented compared to both groups of homozygotes. The effect of genotype on IL-12 and NO production was examined by comparing subjects with each genotype (i.e., alleles 11, 12 and 22), and separately according to the *a priori*
hypotheses, grouping *IL12B*-pro allele-1 homozygotes vs all others, and *IL12B*-3’UTR allele-2 homozygotes vs all others.

Table 13.3. *IL12B* promoter and 3'UTR genotype frequencies in malaria-exposed PNG subjects

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Allele-11</th>
<th>Allele-12</th>
<th>Allele-22</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>IL12B</em>-pro (no. [%])</td>
<td>133 (70)</td>
<td>52 (27)</td>
<td>6 (3)</td>
<td>191</td>
</tr>
<tr>
<td><em>IL12B</em>-3’UTR (no. [%])</td>
<td>38 (20)</td>
<td>68 (36)</td>
<td>82 (44)</td>
<td>188</td>
</tr>
</tbody>
</table>

There was no significant relationship between plasma IL-12 levels and either of the *IL12B* genotypes. Plasma NOx differed significantly according to *IL12B*-pro genotype, with lowest production in *IL12B*-pro allele-2 homozygotes (P=0.007; Figure 13.4). The significance of this effect was maintained after adjusting for age group and sex. There was no difference in plasma NOx according to *IL12B*-3’UTR genotype (Figure 13.5). Neither *IL12B*-pro nor *IL12B*-3’UTR genotype were associated with differences in urine NOx/Cr, NOS activity or estimated 24 hour NOx excretion. Neither *IL12B*-pro allele-1 homozygosity, *IL12B*-3’UTR allele-2 homozygosity or *IL12B*-pro allele-1/3’UTR allele-2 homozygosity were associated with differences in any of the indices of NO production when compared to subjects with other genotypes.
Figure 13.4. Relationship between *IL12B*-pro genotype and plasma NOx (μmol/L) in PNG subjects

The width of each box is proportional to the number of observations in each group.
IL12B-3'UTR Genotype

2
3
4
5

Natural logarithm of plasma NOx

Figure 13.5. Relationship between *IL12B-3'UTR* genotype and plasma NOx (µmol/L) in PNG subjects

The width of each box is proportional to the number of observations in each group.

13.3.3.3. NOS2 polymorphisms

Genotyping was successful for the (CCTTT)$_n$, G-954C and C-1173T polymorphisms in 204, 215 and 212 subjects respectively. The G-954C and C-1173T polymorphisms were absent from the DNA of all subjects genotyped. The (CCTTT)$_n$ alleles were bimodally distributed with peaks at 11 and 13 (Figure 13.6), and the summed number of CCTTT repeats (Figure 13.7) was normally distributed around a mean of 24 (SD 2.8). The GMs for plasma NOx and PBMC NOS activity were 37 µmol/L (95% CI 34-40) and 1020 pmol/mg (95% CI 920-1130) respectively.
Since only 2 subjects were homozygous for “short” (CCTTT)$_n$ alleles = 10 repeats [284], it was not possible to statistically compare NO production in these subjects with others. The summed number of CCTTT repeats [284,286] was not significantly
related to plasma NOx \( (r^2=0.005; P=0.38; \text{ Figure 13.8}) \) or PBMC NOS activity \( (r^2=0.002; P=0.56; \text{ Figure 13.9}) \). Neither measure of NO production was different in those with the \((\text{CCTTT})_{14}\) allele \([428]\) (n=23; \(P>0.5\) for both comparisons) or in subjects with at least one allele of 15 repeats \([286]\) (n=46; \(P>0.8\) for both comparisons). In a post-hoc analysis, there was no difference in NO production in subjects in whom both alleles were 11 CCTTT repeats (i.e., equivalent to the lower bimodal peak defining shorter alleles in this population; n=35; \(P>0.25\) for both comparisons). The potential covariates age, sex and asymptomatic parasitemia were examined in multivariate linear regression models but did not significantly influence the results.

![Figure 13.8. Association between summed \((\text{CCTTT})_n\) alleles and plasma NOx in PNG subjects](image)

203
13.3.4. Discussion

13.3.4.1. Plasma IL-12 levels, parasitemia and \textit{IL12B} polymorphisms

This study has demonstrated a strong inverse correlation between \textit{P. falciparum} parasitemia and plasma IL-12 levels of similar magnitude to that previously found in a study of uncomplicated and severe malaria cases ($\rho = -0.51$) [290] and consistent with the suggestion that background IL-12 expression in whole-blood might be associated with absence of parasitemia in a malaria-exposed population during apparent health [430]. The strength and direction of this relationship validates the borderline significance of the finding that IL-12 was lower in subjects with \textit{P. falciparum} than those without \textit{P. falciparum} parasitemia, which was made in one of three pairwise comparisons. There are a number of plausible explanations for these findings that relate both to cause and effect. \textit{P. falciparum} might suppress IL-12 production directly secondary to impairment of mononuclear cell function by ingested malarial hemozoin [128,290] or indirectly via IL-10 and/or TGF-β production as proposed to explain the lower production of IL-12 in severe compared
to mild malaria [291]. Alternatively, although protozoan GPI molecules similar in structure to the proposed GPI toxin of *P. falciparum* can induce IL-12 production via binding to monocyte TLR-2 [261,432], repetitive stimulation may lead to down-regulation of IL-12 in a process akin to endotoxin tolerance without necessarily relying on IL-10 or other paracrine mediators [433]. This possibility has particular relevance in the context of the present study given that many of its subjects were malaria-tolerant at the time of examination and the evidence against other proposed tolerance-mediating mechanisms such as anti-GPI antibodies (Chapter 14).

Although plasma IL-12 levels were lower in children with severe compared to mild malaria in Gabonese children [290], basal levels upon recovery in the same children were similar thus arguing against an important role for constitutive inter-individual differences in that study. In contrast, genetic differences were thought more likely to explain the *ex vivo* correlation between mitogen and *P. falciparum* antigen-stimulated whole-blood IL-12 activity than exposure to other infections in another study [430] and could at least in part account for the unexplained variance in plasma IL-12 levels in the present study. This is consistent with the notion that IL-12 (or cytokines it induces such as IFN-? [293]) plays an important anti-parasitic role in malaria [414], whereby an inverse association between high constitutive expression of IL-12 and lower levels of parasitemia would be expected. That plasma IL-12 levels in PNG were not associated with either of the *IL12B* polymorphisms previously associated with actual or predicted functional differences in IL-12 expression in Tanzania is not surprising given the geographical separation of the populations and the possibility that linkage disequilibrium explained the Tanzanian findings. These same reasons, along with differences in background stimuli of NO production, could explain these polymorphisms’ non-association with NO production in the present study. The weak but significant inverse correlation between IL-12 and PBMC NOS activity shown in the present study was the opposite to what was expected given that IL-12 is generally regarded to up-regulate NO [97], however, this could be consistent with an inhibitory paracrine effect of NO on IL-12 expression in line with other evidence [434,435].

Although the inter-relationship between IL-12, other immuno-regulatory cytokines and malaria is an extremely complex one, interpretation of the present data in the
context of other studies [290,291,430] suggests that IL-12 production is highest in asymptomatic malaria-exposed subjects and then decreases in proportion to the level of parasitemia encompassing both the tolerant state and clinical disease. Although NO may be involved, it is impossible to be certain on the basis of present evidence exactly how this phenomenon is mediated, with evidence for and against a number of alternative propositions. In this light, the results of this study can be viewed as complementary to those combining data from whole-blood assays and diseased subjects in forming new hypotheses that can preferably be tested in prospective studies including subjects with good health (with and without parasitemia) through to severe disease.

13.3.4.2. NOS2 and IL12B polymorphisms and nitric oxide production

The two single nucleotide NOS2 promoter polymorphisms previously linked with malarial disease severity in Africa (the G-954C [280] and C-1173T [285]) were not present in the PNG cohort. Furthermore, the distribution of CCTTT repeat lengths differed from what had been reported previously in Africa and Thailand [284,286,317] with an increased frequency in the PNG population of (CCTTT)\(_{11}\) and (CCTTT)\(_{13}\) alleles. Most importantly, there was no association between the number of CCTTT repeats and basal NO production in a population whose basal plasma NOx and NOS activity exceeded that which was found in Darwin controls and had previously been reported in non-malaria exposed US controls [298]. Although the measures of plasma NOx and NOS activity potentially incorporated constitutive NO production by other NOS isoforms, NO production from these other sources is likely to be minimal in the context of the high PBMC NOS2 expression demonstrated in tropical populations [96,97]. Given the contradictory nature of the previous NOS2 studies and the absence of functional effects in this study, it is important to reconcile these findings with those of previous reports.

If (CCTTT)\(_n\) microsatellite repeat length independently influences NOS2 transcription, then differences in chronically stimulated basal NO production would have been expected \textit{in vivo}. This was indeed the case with the C-1173T polymorphism in the Tanzanian children [285]. Because there was no relationship between repeat length and NO production in the present study, these data support the
alternative hypothesis - that CCTTT repeat length does not independently influence NOS2 transcription. This is further supported by the following observations: results of clinical studies are conflicting, with severe malaria associated with “short” CCTTT repeats [284], long CCTTT repeats [286] or not at all independently associated with (CCTTT)_n [280,317]; basal and cytokine-induced in vitro NOS2 promoter activity was unaffected in luciferase constructs with deletions in the CCTTT repeat region [429]; and finally, the number of post-hoc analyses in genetic association studies may be under-appreciated and the significance of positive associations may not withstand statistical correction for multiple comparisons.

The mutated G-954C allele frequencies in malaria-endemic sub-Saharan African populations have varied from 7-15% [280,317], compared to only 1-4% in low-endemicity Thais [286] and in an unidentified PNG population that included subjects with malaria [280,286], and an absence from Germans [283] and US whites [317]. The C-1173T polymorphism was present with an allele frequency of 4% in Tanzanian adults and western Kenyan children, but absent from US whites [285]. Selective pressure for these polymorphisms may be altered by variation and fluctuation in malarial transmission intensity on a geographical and temporal basis, as well as exposure to multiple other diseases. The presence and prevalence of genetic polymorphisms may also vary among populations depending on factors other than selective pressure, including founder effect, migration, population sub-sampling and various socio-cultural factors that influence purely random mating. Thus, it is not surprising that there are major differences among populations in the genes controlling immunity to malaria, especially given the potential for polymorphisms in other genes influencing anti-malarial immune responses and the necessity for balance in adaptation to other diseases.

The G-954C polymorphism has been associated with higher basal NOS activity in cultured PBMCs, but did not appear to be associated with significantly increased NOS activity in freshly collected PBMCs ex vivo, in which NOS activity was 10-fold higher than in the cultured cells [280]. Although associated with protection against disease in Gabon [283], no association was found between the presence of the G 954C G-allele and either malaria disease severity or NO production in Tanzanian children [317]. The C-1173T polymorphism was associated with increased basal in vivo
in vivo NO production and a substantially reduced risk of clinical malaria in Tanzanian children, and of severe malarial anemia in the Kenyan children [285]. The absence of the G-954C and C-1173T polymorphisms from our PNG population emphasises that selection of particular NOS2 polymorphisms protective in African populations is not universal among highly malaria-exposed populations, and precluded assessment of functionality in this setting.

There were no significant differences in NO production relating to the two IL12B polymorphisms according to the a priori hypotheses. Interestingly, plasma NOx was significantly decreased in IL12B-pro allele-2 homozygotes compared to other subjects. This would suggest that possession of at least one IL12B-pro allele-1 leads to a higher plasma NOx in PNG subjects, which is the opposite to what was found in Tanzania. While this finding demands further investigation, it should be interpreted with caution given the small number of subjects who were homozygous for IL12B-pro allele-2 (n=6) and the lack of association with other measures of NO production.

13.4. Conclusion

The results of case-control genetic association studies are a potentially powerful and efficient tool for identifying key elements of complex pathophysiological systems, but must be interpreted with caution. Particular attention should be paid to the possibility of reduced statistical power that may arise from multiple pairwise comparisons. If under-appreciated, such statistical inaccuracy may increase the risk of apparently promising new hypotheses leading to fruitless follow-up studies. Conversely, parallel disease and functional associations should lead to new hypotheses being vigorously pursued. The potential for misinterpreting studies of the (CCTTT)_n polymorphism is magnified by the fact that most populations span a range of approximately 10 different allele lengths, all of which can be tested against an outcome in addition to multiple cut-offs defining “higher” from “lower”. It is not surprising then that some very dubious post-hoc associations have been reported [436] and it is quite possible that both of the disease associations described in relation to malaria were due to chance [284,286].
In the Tanzanian study, *IL12B*-pro genotype was assessed at 3 levels (comparing the effect of each genotype; and then segregating the genotypes based on homozygosity for allele-1 and allele-2 in separate analyses) and tested for 2 disease associations (risk of clinical malaria and death from CM). The *IL12B*-3’UTR genotype was assessed at 2 levels, based on the *a priori* hypothesis, and tested for 2 disease associations. A Bonferroni correction for 6 pairwise comparisons and 4 pairwise comparisons would mean that P values less than 0.008 and 0.013 indicate statistical significance respectively. The best model explaining the risk of death from CM was that which included *IL12B*-pro genotype and parasitemia as significant covariates, in which P=0.007. *IL12B* haplotype was significantly associated with death from CM in a non-pairwise likelihood ratio test (P=0.031), and the *IL12B*-pro allele-1/*IL12B*-3’UTR allele-2 haplotype was associated with the highest risk of death in a pairwise test that was one of 4 possible (P=0.007). This would suggest that the positive disease associations identified in this study have a reasonably sound basis statistically and are worthy of further hypothesis-driven investigation.

The parallel finding that *IL12B*-pro genotype and NO production were significantly associated in Tanzanian children, while not evidence of cause and effect, suggests that IL-12 expression can help to maintain NO levels to some degree during clinical disease at a time when overall systemic and PBMC NO production is diminished. This effect was seen independent of the possibility that IL-12 production was decreased in children with CM, which has previously been observed in Gabonese children with severe malaria [290]. NO has been postulated to play a protective [417], if not dichotomous [437] role in human malaria, therefore it is plausible that the amount of NO produced in the face of impending death may influence that outcome. A study of Gambian children [284] (discussed below) also supports that possibility. The lack of association between known homozygosity for the *IL12B* allele-1/2 haplotype and NO production may suggest a lack of cause and effect between death from CM and NO production and/or statistical error due to misclassification of subjects heterozygous at both loci as not having the haplotype. Taken together, these results suggest that measures of NO production should be combined with other measures of IL-12 expression in future studies based on the hypotheses generated by the present study.
How then should the present results be interpreted? First, these results highlight the importance of subjecting hypotheses derived from post-hoc analyses to prospective analysis in follow-up functional correlation studies. Second, these data emphasise that correlation studies should be directed at uncovering whether the proposed polymorphisms (or linked “critical” polymorphisms) are present in regions that directly influence activation or repression of transcription. Third, this study demonstrates geographical differences in the prevalence of potentially important immunoregulatory polymorphisms in populations of similarly intense malaria transmission, suggesting that other undefined \textit{IL12B} and \textit{NOS2} promoter polymorphisms may be functionally important in the PNG population. Alternatively, it is possible that immune responses related to disease severity in one geographic region may be less important in others where host genetics, parasites and malaria epidemiology may differ. Finally, these results suggest that the NO analyses described in Chapter 12 were not significantly confounded by any effects of known \textit{NOS2} promoter and \textit{IL12B} genotypes.

14.1. Introduction
There were two prevailing but independent hypotheses to explain the maintenance and age-dependence of tolerance in malaria-exposed populations prior to the recruitment of the PNG cohort (Chapter 12.2.3). One was production of NO, and the other was neutralising antibodies to the proposed malaria toxin GPI. Support for the latter hypothesis was initially derived from \textit{in vitro} studies demonstrating neutralisation of TNF-α inducing activity of \textit{P. falciparum} by monoclonal antibodies recognising GPI [148,229] and serum from malaria-infected patients [157]. The available evidence suggested that serological cross-reactivity was exhibited by antibodies to \textit{P. falciparum} and \textit{P. vivax} and that the response was predominantly IgM mediated, short-lived and T-cell independent but that longer-lived IgG antibody production could be stimulated in mice by use of adjuvants (Chapter 5.1). Thus it was theorised that children produced high level T-cell independent IgM responses to \textit{P. falciparum} GPIs in proportion to the density of parasitemia and that the subsequent pattern of antibody production paralleled the age-dependent parasite density/pyrogenic threshold curves [190]. Attempts to measure antibodies to \textit{P. falciparum} GPIs in humans had produced results that were rudimentary, in part contradictory and not particularly informative (Chapter 5.1.3).

The opportunity to measure human antibodies to \textit{P. falciparum} GPIs in the PNG cohort was facilitated by their biochemical characterisation and purification in late 2000 [140]. Because antibodies to GPI could potentially decrease disease severity in addition to (or rather than) mediating tolerance, understanding their production and role(s) in chronically malaria-exposed populations is a pre-requisite to the introduction of targeted anti-toxic preventive and therapeutic strategies. The advantages of measuring anti-GPI antibodies in the PNG cohort were that the pattern of antibody production could be accurately defined in relation to age without being confounded by changes associated with acute disease responses and that the
longitudinal design enabled the persistence of responses to be determined. The aim of the study presented in this chapter was to test the prevailing hypotheses related to anti-GPI antibody production at the time, particularly that:

- Residents of highly malaria-endemic populations make antibodies to *P. falciparum* GPIs in response to infection with *P. falciparum* and also *P. vivax*.
- Anti-GPI antibodies are short-lived, with a predominance of IgM over IgG.
- Anti-GPI antibodies show the same age dependence as tolerance.
- Levels of anti-GPI antibodies are proportional to parasitemia, therefore children are expected to have higher antibody levels than adults.
- Alternatively, either or both of these proposed mechanisms could show an age-dependence that parallels resistance to severe manifestations of malaria (i.e., increasing with age rather than decreasing with age).

These hypotheses were tested by measuring IgG and IgM responses against GPIs in different age groups and investigating the association between antibody production and parasitemia both cross-sectionally and longitudinally.

14.2. Methods

Antibodies to GPI were measured in the plasma collected from the study population described in Chapter 12.2.3. For the purposes of the present study, “baseline” samples represented those collected at \(T_0\) (as defined in Chapter 12.2.4) and also included samples from some of the 16 subjects whose initial venesection was cancelled due to rain (Chapter 12.3.1.4), provided that they had not received antimalarial treatment at \(T_0\). “Follow-up” samples were those collected at \(T_2\) (as defined in Chapter 12.2.4). Exclusion criteria were the same as those described previously (Chapter 12.2.4), with the exception that subjects with diarrhea or recent use of non-steroidal anti-inflammatory medication were not excluded from consideration of antibody levels. Statistical analyses were performed and are presented as described in Chapter 10.
14.3. Results

14.3.1. Baseline characteristics

Samples from $T_1$ were unavailable for 9 of the 16 subjects who were given antimalarial treatment at $T_0$ after their blood collection was washed out, in addition to 1 other subject without an available blood sample from $T_0$. Twenty of the remaining 206 subjects met the exclusion criteria (axillary temperature $= 37.5^\circ C$ in 8 subjects; recent malaria history in 14). Three axillary temperature readings were available from 151 subjects; 2 from 29 subjects and 1 only in 6 subjects. Characteristics of the 186 subjects included in this study are given in Table 14.1. After cross-checking and examination of the second smear, one or more additional parasite species were found in 21 of 77 (27%) initially aparasitemic subjects and in 25 of 109 (23%) subjects who were parasitemic on their screening smear.
Table 14.1. Baseline characteristics of 186 PNG subjects in whom anti-GPI antibodies were measured

| Age gp. | Mean Age (yrs [95% CI]) | n | % | P.f | P.v | P.m | P.o | P.v | P.m | P.o | P.falciparum† | n | % | P.f/µL‡ |
|--------|-------------------------|---|---|-----|-----|-----|-----|-----|-----|-----|-----|---------------|---|---|--------|
| 1-4    | 2.8 (2.4-3.3)           | 19 | 26.3 | 9 | 3 | 0 | 0 | 0 | 4 | 0 | 0 | 3 | 12 | 63.2 | 1199 |
| 5-9    | 7.2 (6.7-7.7)           | 36 | 50.0 | 13 | 7 | 3 | 2 | 0 | 5 | 0 | 1 | 0 | 5 | 25 | 69.4 | 431 |
| 10-14  | 11.8 (11.3-12.3)        | 37 | 54.1 | 10 | 6 | 4 | 1 | 1 | 4 | 1 | 3 | 0 | 7 | 22 | 59.5 | 281 |
| 15-19  | 17.0 (16.2-17.7)        | 23 | 39.1 | 11 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 7 | 16 | 69.6 | 163 |
| 20+    | 31.6 (29.1-34)          | 71 | 46.5 | 20 | 3 | 0 | 1 | 0 | 9 | 2 | 1 | 1 | 34 | 24 | 33.8 | 103 |

* Number of subjects with each species (or combined species) of parasite on examination of two consecutive daily blood smears. P.f = P. falciparum; P.v = P. vivax; P.m = P. malariae; P.o = P. ovale; Neg = Negative for parasites.

† Number and percentage of subjects with P. falciparum parasitemia either as the sole infecting parasite or in combination with other parasites.

‡ P.f/µL is the geometric mean of the highest density Plasmodium falciparum parasitemia measured from 2 consecutive daily blood smears.
Follow-up samples taken at a median of 6 weeks (IQR 5-8; range 4-11) from baseline were available from 115 (62%) of the 186 subjects, 9 of whom met the exclusion criteria at this time-point and were excluded from longitudinal analysis. Age, gender and baseline *P. falciparum* positivity did not differ significantly between the groups for whom a second venous sample was or was not available.

**14.3.2. Prevalence of anti-GPI antibodies**

The proportion of subjects positive for IgG antibodies to GPI increased with age (Figure 14.1). Anti-GPI IgG seropositive subjects (median age 19 years; IQR 11-29) were significantly older than seronegative subjects (median age 6 years; IQR 3-12 years; P<0.001). The likelihood of anti-GPI IgG seropositivity was significantly related to age when modelled as a continuous variable (P<0.001): when grouped into 4 ordinal categories according to age (1-4, 5-9, 10-14 and ≥ 15 years), subjects from successive age groups were 3.5 times more likely than their immediate predecessors to be anti-GPI IgG positive (OR 3.5; 95% CI 2.4-5.2; P<0.001). The magnitude of these associations was unaltered after controlling for the non-significant effect of parasitemia with *P. falciparum* and/or other malaria parasites.
Figure 14.1. Percentage of PNG subjects positive for anti-GPI IgG antibodies

The percentage of subjects positive for anti-GPI antibodies is represented by bars and whiskers represent standard errors. The percentage with *P. falciparum* (*Pf*) parasitemia either alone or in combination with other parasites for each age group is represented by diamonds and the geometric mean density of parasitemia by squares (solid line).

Baseline IgM antibody responses were tested in 128 (69%) of the 186 included subjects, as the supply of GPI was limited. IgM antibodies to GPI were absent in all subjects tested who were < 5 years of age and although increasing across successive age groups, IgM seroprevalence was much less than that of IgG (Figure 14.2). After controlling for the significant effect of *P. falciparum* parasitemia (below), subjects ≥ 20 years were 10 times more likely than younger subjects to be IgM antibody positive (OR 10; 95% CI 3.3-30.4; P<0.001).
Figure 14.2. Percentage of PNG subjects positive for anti-GPI IgM antibodies
The percentage of subjects with anti-GPI IgM antibodies is represented by bars, whiskers are standard errors. The percentage with *P. falciparum* (*Pf*) parasitemia either alone or in combination with other parasites for each age group is represented by diamonds and the geometric mean density of parasitemia by squares (solid line).

14.3.3. Intensity of anti-GPI antibody responses
The proportion of subjects with higher level IgG antibody responses increased across successive age groups (Figure 14.3). The level of anti-GPI IgG was positively correlated with age when grouped 1-4, 5-9, 10-14, 15-19, 20-29 and ≥ 30 years (*p*=0.55; *P*<0.001). There was a similar pattern showing higher level IgM antibody responses with advancing age, however, this was not analysed statistically due to the lower numbers of child and adolescent subjects positive for IgM antibodies (Figure 14.4). The intensity of antibody responses relative to Darwin controls was much
stronger for IgG than for IgM in all age groups (see legends; Figure 14.3 and Figure 14.4).

Figure 14.3. Intensity of IgG antibody response to GPI in PNG subjects
The upper limit (mean plus 2 standard deviations) of the responses in 15 non-malaria-exposed Australian adult controls was arbitrarily assigned a value of 1 and antibody responses in study subjects expressed as a multiple of this value (units). Positive responses of increasing intensity are shown in progressively darker shades. Negative responses (Neg) represent adjusted absorbance readings less than the upper limit of the non-exposed controls (i.e., ≤ 1 unit).
Figure 14.4. Intensity of IgM antibody response to GPI in PNG subjects

The upper limit (mean plus 2 standard deviations) of the responses in 15 non-malaria-exposed Australian adult controls was arbitrarily assigned a value of 1 and antibody responses in study subjects expressed as a multiple of this value (units). Positive responses of increasing intensity are shown in progressively darker shades. Negative responses (Neg) represent adjusted absorbance readings less than the upper limit of the non-exposed controls (i.e., ≤ 1 unit).

14.3.4. Cross-sectional relationship between anti-GPI antibody responses and parasitemia

At enrollment, there was no association cross-sectionally between the prevalence of *P. falciparum* parasitemia and the presence of anti-GPI IgG. *P. falciparum* parasitemia was present in 12 (63%) of 19 1-4 year old subjects in the sample but only 1 (8.3%) had IgG and none had IgM. In contrast, 47 (67%) of 71 subjects ≥ 20
years did not have *P. falciparum* parasitemia in their blood smear, yet 42 (89%) had anti-GPI IgGs. Similarly, no association was observed between blood smear positivity for other malaria parasites and anti-GPI IgGs, although these analyses are very likely to have been under-powered due to low numbers (Table 14.1).

In contrast to IgG, anti-GPI IgM seropositivity was associated with *P. falciparum* parasitemia but not associated with parasitemia due to *P. vivax* in subjects ≥ 20 years. In subjects ≥ 20 years, 57% (13/23) of subjects with *P. falciparum* parasitemia (alone or in combination with other parasites) were IgM positive compared to 25% (9/36) without *P. falciparum* (including aparasitemic subjects [OR 3.9; 95% CI 1.3-12; P=0.015]). This relationship was similar after controlling for age (OR 4.4; 95% CI 1.3-14; P=0.03). Excluding subjects with mixed infections, anti-GPI IgMs were present in 56% (10/18) of subjects ≥ 20 years with *P. falciparum* parasitemia compared to 0 of 7 subjects with *P. vivax* (P=0.02). The relationship between anti-GPI IgM positivity and parasitemia was not examined in subjects < 20 years as very few (5 of 69; 7.2%) subjects were positive for IgM antibodies.

14.3.5. Effect of clearance of parasitemia on longitudinal antibody responses to GPI

To further investigate the relationship between anti-GPI IgG seropositivity and parasitemia, paired antibody responses in subjects whose baseline *P. falciparum* parasitemia was cleared by treatment with standard antimalarials (verified on 2 consecutive daily blood smears after 2 weeks) and in whom no recrudescence or reinfection with *P. falciparum* was noted at follow-up were examined. This analysis was further restricted to the 31 subjects with a positive antibody response at baseline, as all 18 seronegative subjects with *P. falciparum* parasitemia at baseline remained seronegative at follow-up.

Eradication of *P. falciparum* parasitemia was associated with a mean fall in IgG antibody responses of 30% (95% CI 17-43%) relative to baseline after a median of 6 weeks (IQR 5-8; P<0.001) in the 31 subjects with an initially positive antibody response. On subgroup analysis, it was evident that this difference was confined to subjects aged = 19 years only. Antibody responses decreased in 17 of 19 subjects
aged = 19 years by a mean of 48% (95% CI 36-60%; P<0.001; Figure 14.5) but were unchanged in 12 subjects aged ≥ 20 years (median increase 1%; P=0.39). There was no change in IgG responses in 26 control subjects without *P. falciparum* parasitemia at either time-point, either overall (mean decrease 3.6%; P=0.55) or in age-based subgroups.

**Figure 14.5.** Longitudinal IgG antibody responses in PNG subjects aged < 20 years with *P. falciparum* parasitemia at baseline

Shown are results from each of 19 subjects whose initial antibody response was positive and whose parasitemia was confirmed to be cleared with standard antimalarial treatment (single dose of sulfadoxine-pyrimethamine).
14.4. Discussion

14.4.1. Relationship between anti-GPI antibodies and parasitemia

This study demonstrates for the first time that, in a cohort of children and adults with intense malaria exposure, the presence of anti-GPI antibodies is directly associated with *P. falciparum* parasitemia. Eradication of asymptomatic *P. falciparum* infection in subjects < 20 years of age was followed by a decrease in IgG antibody responses, whereas there was no change in older subjects. Blood smear positivity with *P. falciparum* was associated with the presence of IgM antibodies to GPIs in subjects ≥ 20 years. Together these observations indicate that *P. falciparum* can induce both IgM and IgG antibodies to its GPIs and that the IgG response is more persistent in adulthood.

There are a number of possible reasons for the lack of a cross-sectional association between blood smear positivity for *P. falciparum* and anti-GPI IgG. Children and adolescents aged 4-14 years from Madang Province have frequent subpatent infections (i.e., PCR positive/microscopy negative) [220] which could induce antibody production, as do adults from a nearby region [21]. Almost all subjects ≥ 15 years were IgG positive, which is likely to reflect increasing persistence of antibody responses between infections, thus making an association with parasitemia more difficult to detect. The association between anti-GPI IgM and parasitemia in subjects ≥ 20 years may have been more evident due to an increased likelihood of IgM responses coinciding with infection, as IgM responses are generally of shorter duration than IgG [223].

Although numbers were relatively small, there was no evidence to support previous suggestions that *P. vivax* induces antibodies that are cross-reactive with *P. falciparum* GPIs [156,157]. In this study, IgM anti-GPI antibody responses were absent in subjects ≥ 20 years with *P. vivax* infection but present in a majority of subjects with *P. falciparum* infection and almost one-third of those who were aperasitemic. The positive responses seen in aperasitemic subjects may reflect recently eliminated and/or subpatent *P. falciparum* infection. In contrast, the lack of IgM response in those with *P. vivax* is consistent with the recent demonstration of species-transcending regulation of parasite density in this region [173]. Although the
mechanism remains unclear [180], it was demonstrated in that study that infection with one species appeared to down-regulate the densities of other species, which resulted in significantly more sequential interspecies infections than concurrent ones.

14.4.2. Age-related differences in anti-GPI antibody responses

The proportion of subjects with positive anti-GPI antibody responses increased with age such that nearly all subjects = 15 years were seropositive for anti-GPI IgG. The intensity of responses continued to increase up to the age of 30 years (data not shown). Another study in Madang villagers aged from 0 to 70 years has shown remarkably similar findings to the present study in the age-related antibody responses to the other blood-stage malaria antigens rhoptry-associated protein (RAP)-1, RAP-2, ring-infected erythrocyte surface antigen (RESA), MSP-1 and MSP-2 [438]. The most noticeable age-related change in both studies was the increase in positive responders that occurred between children aged 0-5 years and 6-10 years [438] or between 1-4 years and 5-9 years (present study). In both cases, this was then followed by an increase in the median levels of antibodies that continued with age beyond 30 years. Similar patterns of age-related reactivity to merozoite surface antigens have been reported in other studies from PNG [439-443] and elsewhere [444,445]. Collectively, those findings relating to relatively conserved malaria antigens support the notion that intrinsic age-dependent differences exist in the ability to mount and sustain antibody responses to malarial antigens. This is also likely to be true in relation to GPI given the intense malaria exposure of subjects in the present study and the likelihood that GPI is structurally conserved in different *P. falciparum* isolates [143].

The existence and nature of intrinsic age-related changes in immune effector functions that ultimately contribute to governing humoral responses to malaria antigens are a key concern for the development of malaria vaccines but are not well understood. This partly reflects difficulties in dissecting the effects of age from cumulative exposure in populations exposed to malaria transmission from birth, as well as resolving acute disturbances to the chronic immune equilibrium that occur during episodes of clinical malaria. In Javanese transmigrants resettling in Papua, individuals developed natural immunity within a year that paralleled that of age-
matched long-term residents with respect to control over the prevalence and density of parasitemia [202]. Recent preliminary data in Javanese transmigrants suggests that the capacity of children aged 6-12 years to raise a detectable antibody response to *P. falciparum* GPIs following sequential symptomatic malaria infections is significantly blunted compared to adults [446]. After convalescence from their first infection and between 2-8 subsequent infections, 50-60% of adults were anti-GPI IgG positive compared to 20-25% of children and persistent antibody responses were uncommon. However, age-related differences have not been shown for all antigens studied in these transmigrants [203,447] and it is possible that variations in exposure behavior or medication use (migrants were routinely supplied with 3 months of chloroquine upon arrival) accounted for some of the differences.

Identifying the mechanisms that underlie the observed age-related differences in immune responses to malaria has proved even more elusive than establishing the phenomenon itself (reviewed by Baird [448]). The capacity to induce intrathymic growth and differentiation of T cells peaks at puberty and this period is associated with the beginning of a gradual decrease in the ratio of naïve to memory T cell subpopulations among CD4+ T cells [448,449]. It has been suggested that this shift in ratio of naïve to memory T cells, combined with an increase in the repertoire of ubiquitous antigens that are recognised with increasing age, enables adults to more effectively stimulate memory T cells [448]. Striking age-related differences in cytokine-producing T cell profiles were demonstrated in malaria-exposed Gabonese subjects aged between 1 and 74 years, with adults displaying a 2-fold higher frequency of IFN-?/-expressing CD4+ cells than infants [450]. An increased frequency of IL-4 and IL-13 producing CD4+ cells was also observed in that study, with the latter being proposed to facilitate a class switch toward IgG1 synthesis. IFN-? has also been postulated to regulate B cell function by skewing the IgG subclass response to malaria antigens toward IgG3 in adults compared to children [451], which has been observed in a number of studies [451-455]. Another consideration that may be relevant to GPIs is that antigens possessing a carbohydrate moiety as an integral component may be less immunogenic in young children than in older children and adults [456]. However, this would seem unlikely to explain the present findings as the ability to respond to polysaccharide antigens is acquired relatively
rapidly, such that children of > 18 months of age would normally be expected to generate an antibody response comparable to that of adults [457].

14.4.3. Inter-individual differences in anti-GPI antibody responses

The variable frequencies in antibody responses to malaria antigens that are observed between individuals at one point in time are likely to in part be explained by variation in the presence and level of antibody responses that occur within individuals over time [458]. Antigenic polymorphism (representing fixed genotypic differences between parasite strains) and antigenic variation (representing gene switching by a single strain of parasite) are other potential sources of inter-individual variation but do not explain differences in response to relatively conserved antigens. The fact that monozygotic twins can differ in their responses to malaria antigens is highly suggestive that environmental factors are just as important as heritable factors in determining humoral immune responses [459,460]. This has led to the theory that clonal dominance by high affinity memory B cells (generated at random in response to early infections in a process termed “clonal imprinting”) over naïve B cells explains much of the person to person variation in response to malaria antigens [459].

In populations with equivalent exposure to malaria, genetic heterogeneity is likely to at least in part account for the remaining variation in responses between individuals. Familial aggregation of total IgG responses [458] and IgG subclass responses [461] toward malaria antigens has been described, even after adjusting for the confounding effect of clustering due to common exposure within households [462]. MHC genes have an important role in regulating immune responses to antigens and evidence from some mouse models has shown that immune responsiveness to malaria antigens differs in MHC-disparate strains [459,463]. Particular HLA alleles have been linked to earlier antibody responses to RAP-1 in 5-15 year old children from Cameroon as well as higher responses in older individuals to RAP-2 [444] but the overall contribution of MHC-related polymorphisms to variation in humoral responsiveness to malaria antigens is thought to be weak [459].
Evidence from twin studies has suggested that genetic polymorphisms outside the MHC encoding regions are more likely than polymorphisms within the MHC to mediate differences in heritable immune responses [356,464]. The genes involved may include those that control T cell and B cell receptor expression and rearrangement; immunoglobulin gene rearrangement; cytokines and cytokine receptors; plasma cell secretion of antibody; production of B cell growth factors; immunoglobulin catabolism and other genes as yet unidentified [459]. One such example has been demonstrated in the Fulani people of West Africa who are less clinically susceptible and more immunologically responsive to malaria than neighbouring ethnic groups [465]. Within the Fulani themselves, an IL-4 polymorphism (the IL4-524 T allele) occurs at high frequency and is associated with elevated antibody levels against malaria antigens [465]. It has been suggested that “a causal relationship exists between the activation of IL-4-producing T-cell subsets and production of the anti-Pf155/RESA-specific antibodies in individuals in which immunity has been induced by natural infection” [466]. Similarly, in vitro cellular IL-4 responses induced by RESA in PNG children have been shown to correlate with protection against subsequent clinical malaria [467]. Although these results may or may not be directly applicable to the present study, they do provide a framework in which genetic control over antibody responses can be considered. In addition, studies such as these give an insight into how it might be possible to elicit improved antibody responses by targeting specific aspects of innate immunity with immunomodulatory vaccine components

14.4.4. Duration of antibody responses to *P. falciparum* GPIs

Longitudinally, anti-GPI IgG levels fell sharply over a period as short as 1-3 months following clearance of parasitemia in subjects = 19 years, in contrast to the stability of antibody responses that was seen in older subjects over this same time period. The possibility that saturated binding obscured any real fall in antibody responses in subjects = 20 years appears unlikely given the considerable overlap of absorbances with younger subjects and the fact that within that range, falls were nearly universal in younger subjects but largely absent from older subjects (data not shown). These data are consistent with the general notion that humoral responses to malarial antigens are relatively short-lived and require ongoing antigenic stimulation to be
maintained [174] and support the conclusions of other studies that have demonstrated age-related differences in the duration of antibody responses to various malarial antigens. For example, antibody responses to a dimorphic region of the gp190 merozoite surface protein were less prevalent, of lower intensity and shorter lived in children aged 2-9 years from Mali than in adults aged 18-73 years when measured during the rainy season and again just prior to the subsequent rains 6 months later [468]. Antibody responses to the polymorphic merozoite surface antigen MSP-1 (block 2) and the conserved antigen RAPI have been shown to fall sharply in as little as 1-2 months following curative treatment of malaria in a longitudinal cohort aged 7-51 years from an area with unstable malaria transmission in Sudan [469,470]. The responses in adults were not compared directly to those of children in these 2 studies and the drugs used to cure malaria were not specified. The possibility that the longitudinal antibody responses in the present study may have been due to skewing of IgG production toward short-lived subclasses [471] is addressed in the following chapter.

Another possible explanation for the decrease in anti-GPI IgG responses seen following clearance of parasitemia in children and adolescents may be that the drugs used to clear parasitemia had an immunosuppressive effect leading to a reduction in antibody production. There have been a limited number of in vitro and in vivo studies that have linked sulfadoxine and/or pyrimethamine to suppressive [472-477], stimulatory [478-480] and neutral immunomodulatory responses [474,481,482]. Few of these studies examined the effects on antibody production directly [473,476,477,480] and it is unclear in 3 of those studies to what degree falls in malaria-specific antibody levels were due to an effect of the drug(s) per se as opposed to removal of the antigenic stimulus due to clearance of parasitemia. Total IgG and IgM antibody levels were measured in healthy Chinese military volunteers (age range 17-22 years) in one study prior to, during, and following cessation of weekly dapsone-pyrimethamine chemoprophylaxis for malaria [476]. IgG levels fell by approximately 22% after 7 weeks of therapy and this decrease was sustained 6 weeks following cessation of the drugs (P<0.005) compared to baseline at both time-points). In contrast, IgM levels fell by approximately 11% after 7 weeks on the drug (P<0.05) but approximated baseline values by the second follow-up. While it appears likely that antibody levels fell as a direct effect of the drug therapy, it is also
possible that drug treatment suppressed other sub-clinical pathogens capable of inducing an antibody response. It remains unclear whether the single dose of sulfadoxine-pyrimethamine given in the present study could have similar effects in younger individuals (but not adults) with longer follow-up in some cases, as the nature of the study design did not allow this possibility to be tested.

14.4.5. Nature of antibody response to *P. falciparum* GPIs

Although the age relationship demonstrated in this study for IgG antibodies to *P. falciparum* GPIs is remarkably similar to that of other malaria peptide antigens [438], it is quite plausible that the humoral immune response to *P. falciparum* GPIs is not MHC-restricted at all. Early studies that examined GPI immunogenicity in mice [225] and neutralising activity of human sera from malaria cases against toxin induction of TNF-a [229] concluded that the immune response to *P. falciparum* GPIs was likely to be T cell-independent and predominantly involve IgM antibodies rather than IgG. However, the low prevalence of IgM responses in this study contrasts with those results and may even suggest that the GPI-attached proteins play a role as natural adjuvants in eliciting a predominantly IgG response [230].

Another possibility is that the presentation of *P. falciparum* GPIs to T cells is CD1-restricted rather than MHC-restricted, which could lead to cytokine-mediated induction of cytotoxic T cells and/or T cell-mediated effects on antibody production that may include differential induction of IgG subclasses (reviewed in Chapter 5.1.2). Patterns of IgG subclass induction have been shown to vary in response to different malarial antigens [483], to switch at different ages [451] and variably correlate with protection or susceptibility to clinical malaria [452]. It remains to be seen whether and how CD1-directed immunoglobulin responses vary in humans of different ages and/or according to genetic polymorphisms in the genes controlling the CD1 system, particularly in relation to GPIs. However, on the basis of available evidence, it is plausible to conclude that immune processing of *P. falciparum* GPIs is just as likely to be CD1-restricted as MHC-restricted and that diversity in CD1-directed regulation of immunoglobulin production may in part explain the pattern of immunoglobulin responses observed in the present study.
An obvious explanation for the parallel drawn between the age-related patterns of the immune responses to *P. falciparum* GPIs and other peptide antigens could be that the GPI used to coat ELISA plates in the present study was impure and contaminated with proteins. The solvent extraction procedures used to strip amino acids and purify the *P. falciparum* GPIs were outlined by Naik et al [140]. Purity was assessed by carbohydrate compositional analysis and by thin layer chromatography (TLC) using fatty acid-labelled similarly HPLC-purified GPI. Contamination from non-glycosylated lipids and/or GPI intermediates was also unlikely as these elute at different retention times during HPLC than GPIs. TLC immunoblotting using immune sera from Kenyan adults was used to confirm that the only material that was seropositive in the HPLC-purified fraction was GPI: i.e., no other seropositive contaminants were detected. Similarly, HPLC purified material from uninfected erythrocyte controls was seronegative when screened against the Kenyan adult immune sera. Definitive proof that the immune response that was measured was wholly directed against *P. falciparum* GPIs will need to await screening of a similar sera set to our own with a chemically synthesised GPI molecule that is structurally homologous to those of *P. falciparum*. It is important to note though, that as long as the ELISA antigen contained sufficient amounts of GPI, the conclusions drawn below in relation to the association between anti-GPI antibodies and tolerance remain valid.

### 14.4.6. Anti-GPI antibodies are unlikely to mediate malarial tolerance

The epidemiological observations that preceded the present study, along with data from animal and *in vitro* models, led to the very reasonable hypothesis that tolerance of parasitemia was mediated by antibodies to malaria toxin(s). Consistent with this hypothesis, it was predicted that children produced higher levels of anti-toxin antibodies than adults in response to their higher density parasitemias [190]. As GPIs are the best characterised fever-inducing malaria toxin identified to date, it was hypothesised in the present study that such antibodies, if present, would recognise *P. falciparum* GPIs. However, the age-related pattern of antibody responses was the inverse of mean parasitemia levels observed in the PNG subjects and also of the negative relationship previously demonstrated between age and parasite density in self-reporting febrile cases from Madang [13]. The positive correlation between age
and the prevalence and intensity of anti-GPI IgG and IgM responses to purified GPIs
in the present study is also consistent with a recent report from Kenya [140] and
another from The Gambia [484]. Most importantly, the finding that anti-GPI
antibody responses were uncommon and only present at low levels in strictly-defined
asymptomatic parasitemic children < 5 years but abundant in adults (even those
without parasitemia) suggests that these antibodies are very unlikely to a major
mediator of parasite tolerance.

It was previously reported that “anti-GPI antibody responses correlated with
protection against malaria-related febrile illness” in Kenyan children aged 0.5 to 3.5
years [140]. It appears from the data presented in that publication that axillary
temperature, measured on a continuous scale, was used in the statistical models as
the correlate of febrile illness and that antibody responder category (grouped as
negative, intermittent and positive) was significantly associated with temperature. If
differences in temperature do not translate to protection against febrile illness, then
the conclusion derived from that data is debatable. Another preliminary study
investigated whether anti-GPI antibodies influenced the likelihood of subsequent
fever following an initial infection in Javanese transmigrant children and adults and
concluded that “anti-GPI IgG may play a role in protection from febrile disease in
this study population”, however, the difference detected was not statistically
significant (P>0.05) [446]. Although the present study was not designed to
investigate the association between anti-GPI antibodies and febrile illness or disease,
the fact that very few children tolerant of high level parasitemias were anti-GPI
antibody positive would suggest that other mechanism(s) are more important in
protecting against fever in this age group. Furthermore, it would seem redundant for
adults to produce such high levels of these antibodies given that fever has been
shown to develop at much lower parasitemias in this age group [13].

14.4.7. Potential roles for anti-GPI antibodies in natural immunity to malaria

As the pathophysiology of severe malaria is dependent on cytoadherence and local
cytokine production [11], it is possible that anti-GPI antibodies play a role in
inhibiting disease progression by down-regulating the processes that lead to both of
these events [124,150]. This potential role is consistent with revised epidemiological
interpretations of the nature of clinical immunity to malaria [14,46,485], however, the data from the present study suggest that it would be likely to be more efficient in older children and adults than in children < 5 years who are most susceptible to severe malaria. This would also suggest that other powerful mechanisms exist that limit the severity of malaria in children < 5 years [419], given that only a small minority of children from this age group succumb to severe malaria in Madang [361]. Data will inevitably emerge from prospective studies testing these hypotheses in the near future, however, that which is available from case-control studies to date has shown no association between anti-GPI antibodies and protection from clinical malaria [484].

It is also conceivable that anti-GPI antibodies have an anti-parasitic role if GPIs are accessible to antibodies on the surface of blood stage parasites during the brief period preceding merozoite invasion of uninfected red cells. Our finding that anti-GPI antibodies increase with age concurrent with a decline in parasite density is consistent with, but does little to prove, this possibility. Given that the role of anti-GPI antibodies is currently unclear, the finding that anti-GPI IgG responses to *P. falciparum* GPIs are of lesser duration in children and adolescents than in adults provides another reason for caution against the treatment of asymptomatic parasitemia in these age groups [387,388,486].

### 14.4.8. Anti-GPI based active and passive vaccination studies

The impetus for designing an anti-GPI malaria vaccine was boosted by the “proof of concept” rodent study showing improved survival in rats immunised with a fully synthetic *P. falciparum* GPI glycan [487] and would be significantly aided if a protective role can be demonstrated for antibodies in prospective studies. In that event, the primary goal of active vaccination should be to reduce the susceptibility of malaria-exposed children to severe disease, with a secondary aim being to protect malaria-naïve subjects with no natural immunity (e.g., occupational exposure, travellers). The data from the present study provides insights into the challenges associated with pursuing an active vaccination strategy in human beings given that generating a sustained antibody response in children < 5 years of age would be the highest priority in endemic areas. Fully understanding the events involved in GPI
antigen presentation and processing (Chapter 5.1) and whether they may be modified by adjuvants or immunomodulators [488,489] may help improve vaccine immunogenicity in this age group. Active vaccination against GPI antigens may theoretically interfere with any protective effect of NO against malarial disease severity as *P. falciparum* GPIs have been shown to induce production of NO by macrophages and endothelial cells *in vitro* [149]. Likewise, vaccination may reduce other potentially beneficial cytokine responses induced by GPIs (such as regulation of parasite density by TNF-a [490]).

An alternative approach to active vaccination is passive vaccination with monoclonal antibodies to GPI, which have previously been shown *in vitro* to neutralise the toxicity of whole parasite extracts [148]. This could be of benefit in the management of severe malaria in malaria-exposed children (without significantly effecting GPI-induced production of NO) and also in previously malaria-naïve patients. Although the outcome of CM in Gambian children was not improved by administration of a monoclonal antibody to TNF-a [62], passive vaccination against *P. falciparum* GPIs has the potential advantage of inhibiting the earlier events initiating the cytokine cascade. A similar strategy using polyclonal antibodies that recognise bacterial superantigens has been reported to improve clinical outcomes in streptococcal toxic shock syndrome [491] which shares several features of the Th1 cytokine-dominated inflammatory response to severe malaria [492]. Another therapeutic option would be to inhibit GPI biosynthesis by *P. falciparum* with drugs that target unique plasmodial enzymes, given evidence that the biosynthetic pathway differs significantly from that described in humans and that GPI appears to be essential for parasite maturation [145]. This possibility may be facilitated by the increasingly precise characterisation of the GPI biosynthetic pathway in recent studies [144] along with the identification of *Plasmodium*-specific “suicide substrate inhibitors” [493] and other inhibitory compounds [494]. The identification of a natural compound in a yeast model that inhibited GPI biosynthesis in mammalian lymphoma cells but not protozoa (including *P. falciparum* [495]) lends further support to this idea.
14.4.9. Conclusions

The present study has clearly defined the pattern of anti-GPI antibody responses in a population of strictly defined asymptomatic children and adults whose clinical malaria epidemiology is well characterised. These data show that *P. falciparum* induces antibodies to its GP1s in individuals living in a region of high malaria endemicity that are more easily elicited, of higher intensity and more persistent with increasing age. This pattern is the complete inverse of what would be expected if anti-GPI antibodies were a major mediator of malarial tolerance and suggests that these antibodies could play no more than a minor role in preventing severe malaria in the youngest children. Replication of the current findings using a synthetic form of GPI that is structurally identical to that of *P. falciparum* is important to dispel any remaining doubt that the present findings could have been confounded by protein contamination of the ELISA antigen. The ability to rapidly synthesise GPI molecules using new automated solid-phase oligosaccharide techniques [496] should enable realisation of such studies sooner rather than later.

The pattern of antibody response was similar to that reported for other peptide malaria antigens in the same region. This would be consistent with MHC-restricted regulation of the anti-GPI response or alternatively would suggest that the mechanisms regulating a CD1-directed humoral response are very similar. T cells are likely to be pivotal mediators in both instances and changes in T cell profiles and activity with increasing age could underlie the variation that occurs. Switching of IgG subclass expression may be one consequence of this process. In addition to intrinsic age-related changes, some of the variation in the frequency of antibody responses between individuals of the same age is likely to be under a number of different genetic controls. These could include different HLA alleles in the case of an MHC-restricted antibody response, similar but as yet undefined differences in CD1-restriction and/or polymorphisms in the genes controlling cytokine expression that ultimately regulate humoral responses.

Even though these data argue against a role for anti-GPI antibodies in mediating tolerance of parasitemia, it is possible that anti-GPI antibodies could reduce the severity of malaria in those most susceptible (including young children and malaria-naïve individuals). This could lead to the therapeutic use of a monoclonal antibody
against GPI to passively immunise against severe disease or alternatively, active vaccination. Understanding the reasons for age-related and inter-individual differences in antibody responses should be a key concern for those embarking on such strategies. A particular challenge will be to overcome the short-term nature of antibody responses that were observed in subjects up to 19 years of age.

Finally, whether anti-GPI antibody responses fell following drug treatment due to clearance of parasitemia or drug-induced immunosuppression may be immaterial if reducing antibody levels leads to an increased rate of disease or progression. Because of this uncertainty, caution should be exercised in deciding whether or not to treat asymptomatic parasitemia, which is still standard practice following malariometric surveys in some jurisdictions [321].
Chapter 15. Immunoglobulin G responses to *Plasmodium falciparum* glycosylphosphatidylinositols are short-lived and predominantly of the IgG3 subclass

15.1. Introduction

In the previous chapter, it was demonstrated that that natural human immune response to parasite-purified *P. falciparum* GPIs in the PNG cohort exposed to intense malaria transmission was characterised by an increasing prevalence, persistence and intensity of antibody responses with age. IgG responses decreased significantly in the Madang children and adolescents within as little as 6 weeks following clearance of parasitemia and were significantly higher at the end of the transmission season in a Gambian study than at the start [484]. In the absence of a definitive case-controlled longitudinal study, understanding the limitations of the naturally elicited immune response to GPIs is important in maximising the potential of synthetic vaccination strategies aimed at generating long-lasting immunity. Other studies describing IgG antibody responses to malarial proteins have identified skewing toward particular sub-classes, especially IgG3, as being associated with short-lived activity [471] but much less is known about the nature of antibody responses to GPIs. The aim of the present study was therefore to investigate the reasons for the short-lived anti-GPI IgG responses in the PNG cohort by measuring the GPI-specific IgG subclass response before and after clearance of parasitemia in subjects with *P. falciparum*. As the age-related antibody response to GPIs was very similar to those described in relation to other malaria antigens, it was hypothesised that the anti-GPI IgG subclass response would be skewed toward the more transient IgG3 subclass in children and adolescents but that IgG1 responses may predominate in adults.
15.2. Methods

15.2.1. Subjects

The 31 subjects included in the present study were those from the PNG cohort (Chapter 12.2.3) in whom: no symptoms of malaria or other illness were present at the time of initial examination or at follow-up 6 weeks later; a positive anti-GPI total IgG response had previously been demonstrated; and *P. falciparum* was detected on initial microscopy and confirmed to have been eradicated following treatment with sulfadoxine-pyrimethamine.

15.2.2. Statistical analysis

Statistical analyses were performed and are presented as described in Chapter 10. Subjects = 20 years were classified as adults as per the previous study (Chapter 14).

15.3. Results

15.3.1. Baseline cross-sectional anti-GPI IgG subclass responses

Four subjects were aged 5-9 years; nine were 10-4 years; six were 15-19 years and twelve were = 20 years (median 15 years; IQR 9-32). Fifteen subjects were male. The GM baseline IgG<sub>1</sub> was 1.3 µg/mL (95% CI 0.7-2.3) and IgG<sub>1</sub> levels increased significantly with age in years ($r^2=0.26$; $P=0.003$). The GM IgG<sub>1</sub> in adults was 3.5 µg/mL compared to 0.7 µg/mL in younger subjects ($P=0.004$). The GM baseline IgG<sub>3</sub> was 9.2 µg/mL (95% CI 5.9-14.3), which was significantly higher than for IgG<sub>1</sub> ($P<0.001$; Figure 15.1). IgG<sub>3</sub> levels were also higher in adults (GM 13.7 µg/mL; 95% CI 6.6-28.4) than in non-adults (GM 7.1 µg/mL; 95% CI 4.0-12.7), although this was not statistically significant ($P=0.15$). The ratio of IgG<sub>3</sub> to IgG<sub>1</sub> levels was highest in the youngest age group and decreased significantly with advancing age ($r^2=0.16$; $P=0.028$).
Figure 15.1. Baseline anti-GPI IgG₁ and IgG₃ subclass responses in PNG subjects

Presented are the logarithms (base 10) of IgG₁ (white boxes) and IgG₃ (dotted boxes) antibody concentrations in µg/mL for 31 subjects aged 5-60 years. Horizontal bars represent medians and means are represented by dots. The bottom and top of the boxes are the 25th and 75th centiles respectively; whiskers extend to adjacent values no more than 1.5 times the interquartile range from these centiles and outlying values are represented as circles. The width of each box is proportional to the number of observations. IgG₃ levels were significantly higher than IgG₁ (P<0.001). IgG₁ levels increased (P=0.003) and the ratio of IgG₃ to IgG₁ decreased significantly with advancing age, from 37 times (95% CI 6-240) in the 5-9 year age group to 4 times (95% CI 1.5-10) in adults.

IgG₄ was below the lowest level that could be detected (i.e., 0.06 µg/mL) in all of 13 subjects who were tested (median age 15 years; IQR 13-22). IgG₂ was below the lowest level that could be detected (i.e., 0.02 µg/mL) in 12 of these 13 subjects, and was 0.16 µg/mL in the remaining subject (aged 32 years).
15.3.2. Longitudinal anti-GPI IgG$_1$ and IgG$_3$ responses

Follow-up IgG$_1$ and IgG$_3$ responses were determined after a median interval of 44 days (IQR and range 28-77). The GM IgG$_1$ at follow up was 0.7 µg/mL (95% CI 0.3-1.5), and for IgG$_3$ was 5.6 µg/mL (95% CI 3.3-9.7). IgG$_1$ levels fell in 24 of 31 subjects between baseline and follow-up by a mean of 49% (95% CI 28-64; P<0.001). The mean percentage fall in adult subjects was 17% (95% CI -2-32; P=0.07) compared to 63% (95% CI 37-78; P<0.001) in younger subjects. IgG$_3$ levels fell in 29 of 31 subjects between baseline and follow-up by a mean of 39% (95% CI 28-48; P<0.001). In contrast to IgG$_1$, the fall in IgG$_3$ was significant for both adults (mean fall 32%, 95% CI 13-48; P=0.006) and non-adults (mean fall 43%, 95% CI 29-54; P<0.001).

15.4. Discussion

This study defines the immunoglobulin IgG subclass-specific components of the humoral response to *P. falciparum* GPIs in humans and provides the basis upon which to understand the sharp and rapid decline in total anti-GPI IgG responses that was previously observed [497]. This study demonstrates that the antibody response induced by *P. falciparum* GPIs in subjects repeatedly exposed to infection is characterised predominantly by IgG$_3$, with a lesser contribution from IgG$_1$ and an absence of IgG$_2$ and IgG$_4$. IgG$_3$ responses declined with clearance of parasitemia in subjects of all ages, whereas IgG$_1$ was more stable in adults. Reporting antibody concentrations rather than ODs minimised the potential for misinterpretation that may result from widely different affinities of the secondary antibodies [498]. This also avoided over-estimating the magnitude of change in antibody response that may accompany interpretation of ODs alone given the exponential nature of the OD/concentration curve.

A number of studies examining IgG subclass responses to *P. falciparum* peptide antigens have also demonstrated skewing toward the IgG$_3$ subclass, most particularly against MSP-2 [454,499] but also against other epitopes [451]. The absence of IgG$_2$ and IgG$_4$ responses and the high ratio of IgG$_3$ to IgG$_1$ subclasses observed in the present study is remarkably similar to the pattern of anti-MSP-2 responses previously described in Solomon Islanders [471] even though the antigens involved are
fundamentally different. This contrasts with the IgG\(_2\) and IgG\(_1\)-dominated responses that have been described toward carbohydrate antigens such as bacterial polysaccharides [500] and may indicate that GPI-anchored proteins act as natural adjuvants in eliciting immunity to *P. falciparum* GPIs. Alternatively, GPI molecules might directly induce this pattern of IgG production, which is supported by *in vitro* evidence showing that GPI-related glycoinositolphospholipids from *Trypanosoma cruzi* can induce both IgG\(_3\) and IgG\(_1\) production by murine B cells in the absence of covalently-linked proteins but with a requirement for cytokine co-stimulation [501].

If *P. falciparum* GPIs are subsequently shown to be of key importance in malarial toxicity, then the major aim of immunisation with synthetic *P. falciparum* GPIs will be to generate effective and long-lasting immunity in young children most at risk of severe malaria. In these children, natural anti-GPI responses are frequently absent or unstable despite repeated parasite exposure [140,484,497]. Moreover, in those who do respond, IgG\(_3\) responses predominate, are generally more transient than IgG\(_1\) and are likely to require frequent boosting to be maintained [471]. It is presently unclear how effective such natural antibody responses may be in preventing disease and it is possible that naturally elicited antibodies recognise functionally irrelevant epitopes [484]. On the basis of this evidence, it may be impractical and insufficient to reproduce the natural immune response to GPIs by vaccination in order to protect non-immunes against malaria pathogenesis. Instead, co-stimulatory molecules or appropriately designed adjuvants will probably be needed to induce sustained IgG responses, which may be facilitated by determining whether the subclass responses demonstrated here are MHC or CD1-restricted [234].

### 15.5. Conclusion

This study demonstrates for the first time that the characteristically short-lived natural human immune response to *P. falciparum* GPIs results from a skewing of IgG antibodies to the more transient IgG\(_3\) subclass. Furthermore, if *P. falciparum* GPIs transpire to be of central pathogenic relevance to human malaria, then these data suggest that the aim of vaccination with synthetic *P. falciparum* GPI analogues should be to induce long-lasting effective immunity that substantially differs from the natural response.
SECTION Four.

Concluding synopsis and future directions
How it is that individuals living with malaria have reached a status quo enabling them to ward off the parasite’s damaging effects while at the same time allowing it to continually replicate in their bloodstream is perhaps the most intriguing and least understood aspect of the host-parasite interaction. Given the propensity for *Plasmodium falciparum* to cause symptoms such as fever at low densities in malaria-naïve subjects, the capacity to maintain this equilibrium must either be innate or otherwise established very quickly under the cover of passively acquired maternal immunity in regions of high malarial endemicity. This situation (called tolerance) may have been influenced by genetic selective pressures, but only in so far as the human mechanisms mediating it are required to prevent death or ensure fitness for mating. It is more certain though that these mechanisms operate in a complex microenvironment in which co-evolution of human beings and malaria parasites (and other competing interests) has been going on for many thousands of years. Evidence that tolerance can be induced (by more than one species of malaria parasite) and that it varies with malaria endemicity indicates that it is also, at least in part, acquired. Detailed epidemiological studies spanning more than 60 years have painted a picture of tolerance that reveals one more intriguing aspect - children as young as one year of age, despite having more frequent clinical attacks and being more susceptible to death from malaria, also appear to tolerate much higher levels of parasitemia than adults.

Atop the groundwork laid by epidemiological observations has been constructed an expanding pathophysiological model borne of the initial deductions made by Camillo Golgi over 100 years ago. The characteristic paroxysmal fevers of malaria appear to arise from the host response triggered by a malarial toxin (or toxins) subsequent to the periodic bursting of blood stage schizonts. These events include the release of endogenous fever producing substances such as the cytokine tumor necrosis factor alpha (TNF-a) and interleukin (IL)-6. Interest in candidate malarial toxins has narrowed to the glycosylphosphatidylinositol (GPI) protein anchor, which is attached by lipid chains to the outer parasite cellular membrane. Numerous *in vitro* and animal studies have demonstrated a role for the GPI of *P. falciparum* and other protozoa in TNF-a and fever induction, as well as the initiation of other potentially pathological events in human malaria such as endothelial cytoadherence receptor expression and NO production. This area of research has evolved rapidly to the stage
that GPI analogues can now be readily synthesised biochemically and GPI biosynthesis potentially targeted therapeutically, thus the importance of *P. falciparum* GPIs to human disease should be established in the foreseeable future one way or another. As toxicity can be considered the *sine qua non* of malarial pathology, understanding its biological processes has the potential to inform strategies aimed at reducing death from malaria, as well as the burden of disease.

It is very reasonable to suppose that the mechanisms which mediate tolerance of malaria in humans in some way inhibit the chain of events triggered by the malaria toxin(s). It follows that anti-toxic mechanisms may also act to prevent progression of disease once established, however, untangling their contribution to retarding disease in the presence of numerous concurrent and interacting immunopathological responses is far from straightforward. The prevailing theories on how anti-toxic mechanisms functioned to mediate tolerance prior to this thesis sought to combine epidemiological imperatives (such as age-dependence and impersistence) with the data available to inform hypotheses worthy of testing in humans. The two most likely candidates were NO and antibodies to GPI, based respectively on data from animal and human studies suggesting that NO might ameliorate disease in the presence of parasitemia and on *in vitro* studies demonstrating toxin-neutralising activity of animal and human serum through antibodies recognising phosphatidylinositol. It was proposed that NO, produced in response to malaria infection but possibly also from other sources, participated in the down-regulation of inflammatory cytokine responses in a process akin to that of bacterial endotoxin tolerance. NO had been shown to participate in the events mediating bacterial tolerance and cross-tolerance to the effects of malaria and endotoxin had previously been demonstrated in humans. Antibodies to GPI were presumed to be predominantly of the immunoglobulin (Ig)M class (thus short-lived), suggesting that longer lived memory IgG responses were not being induced given the constant antigenic challenge. In both cases, the general assumption that adults suppressed parasitemia to lower levels than children provided an explanation for the proportionate decrease in anti-toxic effector mechanism with age, given its intrinsic link with induction by malaria. Though not necessarily mutually exclusive, these hypotheses were proposed in parallel.
The purpose of this thesis was to apply the knowledge gained by others to test these two prevailing hypotheses directly in humans chronically exposed to intense malaria transmission and in whom chronic asymptomatic parasitisation was the norm rather than the exception. Although a prospective, active and intense longitudinal surveillance study might have been the ideal for testing these hypotheses, this was well outside the realms of this PhD and possibly outside the bounds of what would be acceptable to a generally healthy population of individuals going about their daily lives. It was feasible though to test cross-sectionally and longitudinally some of the central assumptions underpinning the development of these theories. In particular, the presumed age-dependence of both phenomena, their relationship with parasitemia, the inference of genetic determinism in NO production and of longevity in anti-GPI antibody production. These studies were facilitated by the collaborations established with researchers in Indonesia and Papua New Guinea (PNG), the purification of *P. falciparum* GPI, and the goodwill of two populations on either side of the border in New Guinea who it must be recognised, participated out of true altruism. The first field project was a small cross-sectional pilot study involving adults in Papua province (formerly Irian Jaya) and the second enrolled children and adults from Madang, PNG who were given treatment to eradicate parasitemia and then followed longitudinally to evaluate its effect. Total systemic NO production was estimated along with cellular production by peripheral blood mononuclear cells (PBMCs), thought to be most receptive to induction by infection and high output NO production. Anti-GPI IgM, IgG and IgG subclass antibodies were also determined cross-sectionally and longitudinally.

Systemic and PBMC NO production was generally elevated in both study populations to levels much higher than expected from previous studies in malaria-naïve subjects and evident in healthy Darwin adult controls. Extending previous findings in children, this study for the first time demonstrated elevated NO production/PBMC NO synthase (NOS) activity in asymptomatic malaria-exposed adults. In Papua province, systemic NO was comparable in urban controls (provincial city; low level malaria exposure) and a parasitemic rural subjects, but much higher in asymptomatic parasitemic subjects. Interestingly, PBMC NOS activity was similar in urban and rural subjects regardless of parasitemia. NO production was similarly high in PNG across all ages, i.e., from age 2 to age 60, and
there was no age-dependence in this relationship. There was no cross-sectional
relationship between NO and parasitemia in PNG, and eradication of
microscopically-detectable parasitemia (confirmed by PCR) made virtually no
difference to NO production longitudinally. In fact, within subject variation of NO
production over three time-points spaced over approximately 8 weeks was minimal
despite the “coming and going” of parasitemia.

Weak but significant associations between PBMC NOS activity and both IL-18 and
IgE in different sites provided some clues as to the basis for NO production.
Surprisingly, a negative correlation was evident between plasma IL-12 (generally
thought to be a potent inducer of NO) and PBMC NOS activity, which on the
balance of evidence may have reflected down-regulation of IL-12 by NO in this
setting. The negative correlation between IL-12 and *P. falciparum* parasitemia may
be due to down-regulation of IL-12 as a reflection of endotoxin-like tolerance or
mononuclear cell ingestion of hemozoin. Alternatively, this cytokine may play a
dominant anti-parasitic role during asymptomatic parasitemia - it seems rather
unlikely to be mediating malarial tolerance. Interestingly, as there was no correlation
between systemic NO production and PBMC NOS activity, the data from these
studies suggest that other cellular sources are just as important. Overall, these results
sit comfortably with an emerging picture in which the sum-total of NO production in
populations such as these is influenced not only by malaria but also by other
infections/factors which are likely to be at least as influential. The paradox between
study sites in the relationship between NO and parasitemia is a genuine conundrum,
not amenable to explanation by these results. If results from one site or the other
occurred as a result of chance, then it is the PNG study which should be believed
given the larger numbers and the strength of its longitudinal design. Of the other
possible explanations, the existence of a ceiling to NO production which is exceeded
in PNG all of the time through background non-malarial stimulation but not in Papua
province (e.g., due to lower rates of intestinal parasitosis) is the most amenable to
testing.

The carefully controlled study design in PNG, which minimised the potentially
confounding effect of dietary nitrites and nitrates on estimation of NO and is unlikely
to have been affected by renal dysfunction, provided an opportunity to test the
influence of genetic polymorphisms in the NOS2 and IL-12 genes on basal NO production in a malaria-endemic setting where chronic stimulation of NOS2 results in high basal NO production. In this sense, this study was unique in enabling the “missing link” between polymorphism and outcome in disease association studies to be tested under field conditions that accurately reflected the influence of other factors and processes common to malaria-endemic environments. The polymorphisms tested had all previously been linked to disease outcome ± altered NO production. Two single-nucleotide NOS2 gene polymorphisms were not present in PNG and the prevalence/pattern of polymorphisms in the IL-12 promoter and of a microsatellite pentanucleotide repeat (CCTTT) differed from that previously described in Africa. Neither of the expected associations were shown with these polymorphisms. In the case of the IL-12 polymorphisms, it is impossible to be certain whether the results from Africa or PNG were due to chance or just reflected differences in gene linkage or regulation. In the case of the CCTTT polymorphism, it is possible that linkage to other polymorphisms explained the results from other studies in Africa and Thailand. However, the opportunity for investigators to do multiple statistical comparisons on a polymorphism with over 10 allele sizes, in which single alleles, cut-offs between high and low and summation of allele lengths can all be tested in association with an outcome is immense, and may be irresistible. The fact that the two disease association studies of CCTTT showed diametrically opposite but positive associations and that no link with NO was found in the PNG study suggests that CCTTT repeat length may be of little biological importance. Profoundly important genetic associations are unlikely to be subtle, and great care should be exercised in the interpretation of genetic association studies in the absence of functional data to support their assumptions.

It was very clear, and has subsequently become clearer from African studies, that antibodies to *P. falciparum* GPIs are strongly related to age, but not in the way that was previously supposed. The prevalence, level and persistence of anti-GPI IgG in PNG subjects all increased with age and IgM antibodies were uncommon. Antibody responses fell with clearance of parasitemia in children and adolescents but were more stable in adults. The reason for this appeared to be skewing toward production of the shorter lived IgG₃ subclass, which has also been noted in response to other malarial antigens and is a problem that can potentially plague successful vaccination.
strategies. It is important to note that these conclusions would be supported by confirmation of these findings in studies using fully synthetic GPI without the possibility of contamination by other parasite products. Nevertheless, by demonstrating what individuals living under intense exposure to malaria are capable of, these results could prove useful in directing the types of responses to be induced by anti-GPI vaccines and identifying hurdles that may be overcome with adjuvants. While animal data is encouraging and it is possible that an approach targeting GPI could prove useful in limiting disease severity, it appears extremely unlikely from the PNG data and that from elsewhere that anti-GPI antibodies mediate the phenomenon of tolerance.

It needs to be said that the conclusions of this thesis could be irrelevant if the epidemiological assumptions underpinning tolerance are incorrect (e.g., if peripheral parasitemia did not accurately reflect parasite biomass during good health due to large age-dependent differences in sequestration). However, while the alternative hypotheses may give some comfort to those seeking not to concern themselves with the existence of tolerance, they seem no more plausible than the obvious conclusions derived by malariologists going back more than half a century. In this respect, it is possible that the growing understanding of parasite dynamics and (by exclusion in the case of this thesis) the conceptualisation of anti-toxic immunity will converge to facilitate the testing of other hypotheses. The most obvious conclusions from this thesis are that: tolerance may develop in a process akin to endotoxin tolerance, just not depend on NO; that age-dependence, if it exists, is due to hitherto unknown differences between children and adults that may have an evolutionary basis (i.e., people do what they can when they can to stay alive, and in the absence of acquired mechanisms, those with innately tuned protective mechanisms do survive); that naturally occurring anti-GPI antibodies do not mediate tolerance, and while a role in retarding disease pathogenesis cannot be excluded, they may recognise functionally irrelevant epitopes of the molecule.

Other potential roles of NO in anti-toxic (and anti-parasitic) immune responses to malaria do require further testing given the potential for NO to reduce disease severity. It must be recognised though that the association of NO with improved outcomes in clinical studies is just that, not necessarily causal. Evidence for a causal
association comes mainly from the small number of genetic associations studies and no universally important polymorphism has yet been determined. Prospective studies with active surveillance, although laborious, may be needed to clarify this situation. Alternatively, intervention studies predicted to do little harm (e.g., administration of the amino acid arginine, the substrate for NO production) in controlled environments could reveal a beneficial effect but the theoretical possibility of increasing NO-mediated pathology must be realised and minimised. The assumption that other infections (such as intestinal parasitosis) could be the reason for the very high NO production seen in the studies outlined in this thesis could be easily tested by measuring NO before and after anti-helminthic treatment, but on the basis of present evidence may satisfy only a scientific curiosity rather than a pressing need. The drive for an anti-toxic malaria vaccine was boosted by the rodent study showing a reduction in mortality in mice immunised with synthetic \textit{P. falciparum} GPI glycan from the rodent malaria strain \textit{P. berghei}. However, the mice in whom infection was induced in that study share few similarities with the chronically parasitised subjects in the studies described in this thesis for whom a vaccine is most desirable. In addition to the need to develop a long-lasting immune response, particular care needs to be paid to the very real possibility that cross-reactive antibodies could be induced to the vaccine epitope that recognised human GPI and resulted in autoimmune disease. Furthermore, an anti-toxic vaccine may do little to halt increasing parasite replication, therefore combination with epitopes that aimed to induce anti-parasitic immunity may be essential for this to be a useful approach. Perhaps then, passive vaccination of an experimentally infected adult with anti-serum raised to synthetic \textit{P. falciparum} GPI would provide the most efficient and least harmful means by which to take the next step in advancing the development of what might become the first efficacious vaccine against human malaria.
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