An investigation of the relationship between malaria and red-cell polymorphisms in Madang, Papua New Guinea.

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November, 2007.

This work is submitted in partial fulfilment of the requirements of Oxford Brookes University for the degree of Doctor of Philosophy on the basis of published work.

Contents

List of illustrations

2. O'Donnell A, Weatherall DJ, Taylor AM, Reeder JC, Allen SJ. Muscle cell injury, haemolysis and dark urine in children with falciparum malaria in Papua New Guinea. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2006; 100: 817-825.

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4. O'Donnell A, Raiko A, Clegg JB, Weatherall DJ, Allen SJ. α^{+}-thalassaemia and pregnancy in a malaria endemic region of Papua New Guinea. *British Journal of Haematology* 2006; 135: 235-241.

5. O'Donnell A, Allen SJ, Mgone CS, Martinson JJ, Clegg JB, Weatherall DJ. Red-cell morphology and malaria anaemia in children with Southeast Asian ovalocytosis band 3 in Papua New Guinea. *British Journal of Haematology* 1998; 101: 407-412.

6. Allen SJ, **O'Donnell A**, Alexander NDE, Mgone CS, Peto TEA, Clegg JB, Alpers MP, Weatherall DJ. Prevention of cerebral malaria

by Southeast Asian ovalocytosis in children in Papua New Guinea. *American Journal of Tropical Medicine and Hygiene* 1999; 60: 1056-1060.

7. O'Donnell A, Raiko A, Clegg JB, Weatherall DJ, Allen SJ. Southeast Asian ovalocytosis and pregnancy in a malaria endemic region of Papua New Guinea. *American Journal of Tropical Medicine and Hygiene* 2007; 76: 631-633.

Acknowledgements

I would like to express my sincere thanks to Professor Sir David Weatherall for giving me the opportunity to undertake this fascinating programme of research and for his constant support, encouragement, supervision and guidance. I would like to thank Professor John Clegg for his guidance in laboratory procedures, patience and close attention to detail.

I would like to acknowledge the support of Dr Michael Alpers and his staff at the Papua New Guinea Institute of Medical Research who helped to facilitate this research programme and to Dr Andrew Tuka, College of Health and Allied Sciences, Madang, for providing laboratory space in the most idyllic setting; on the cliff-tops overlooking the Bismark Sea! Special thanks go to Martha Mellombo, Andrew Raiko and Willie Depsone for their invaluable help in the laboratory and hospital and with fieldwork. I would also like to thank the women and children of Madang, without whose participation, these studies would not have been possible.

I am very grateful to Professor Julian Hopkin and Professor Gareth Morgan for providing me with laboratory and office facilities in The School of Medicine, Swansea, to complete this programme of research.

At Oxford Brookes University, I am very grateful to Dr Susan Brooks for supervision, advice and very helpful comments with the preparation of this thesis

and to Dr David Evans for his advice and support regarding registration and submission of this thesis.

Finally, my sincere thanks go to my husband, Stephen, for his encouragement and helpful comments - not to mention an immeasurable amount of patience and support! I would like to dedicate this work to my three lovely daughters, Stephanie, Sophie and Holly and thank them for their understanding.

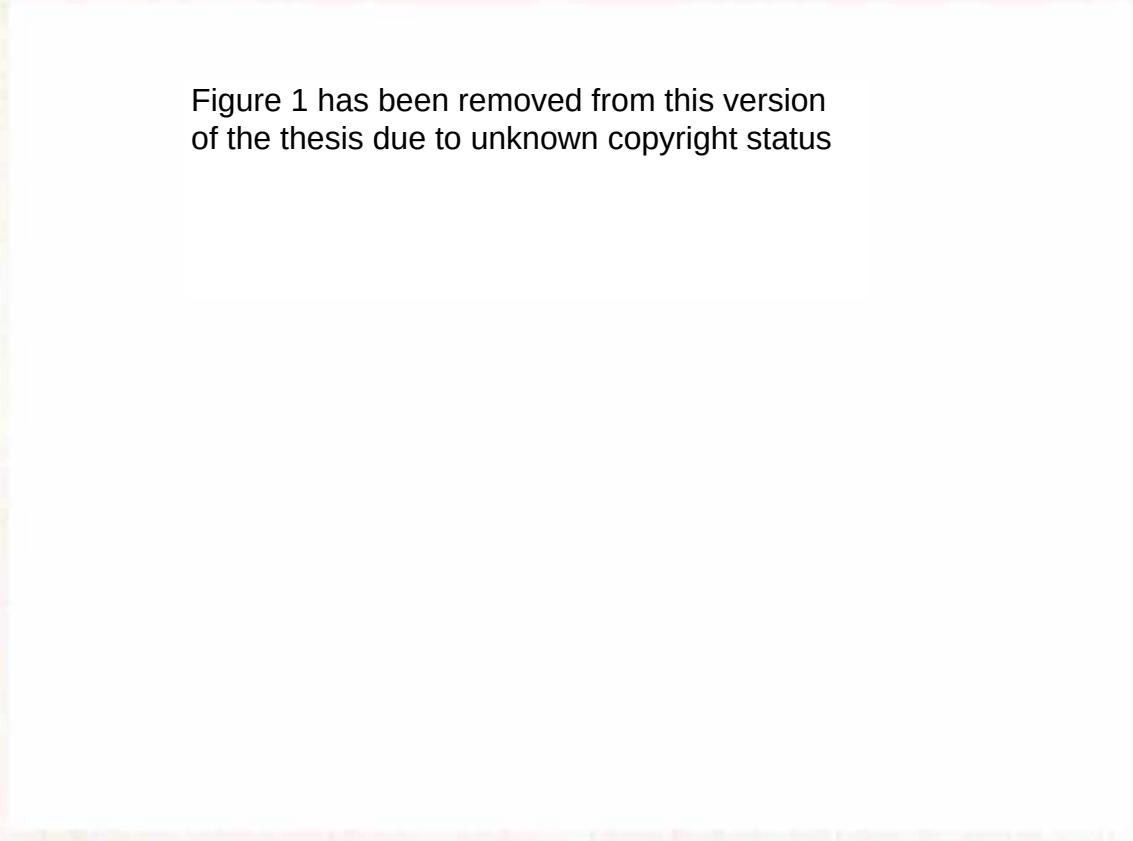


Figure 1: Papua New Guinean children in traditional dress.

Abstract

In 1949, JBS Haldane proposed that the high frequency of the thalassaemias might reflect a heterozygote survival advantage against malaria. This "malaria hypothesis" formed the basis for this programme of research. Between 1993 and 2006, I conducted a series of comprehensive and detailed studies of the relationships between malaria and the common red-cell variants α- thalassaemia and Southeast Asian ovalocytosis (SAO) in children and pregnant women in the North coastal region of Papua New Guinea.

The clinical and laboratory features of malaria in children were described. In multiple regression analysis, raised plasma lactate (> 5mmol/l) was the major predictor of death. Acidosis and impaired renal function were also independent predictors of mortality, and muscle-cell injury was identified as a cause of dark urine.

A case-control study of 249 children admitted to hospital with severe malaria and 233 children with severe non-malaria illnesses was undertaken to investigate the possible protective effect of α^+-thalassaemia and SAO against the severe manifestations of malaria and other severe illnesses. Homozygous α^+-thalassaemia reduced the risk of severe malaria by 60% and SAO appeared to provide complete protection against cerebral malaria. Interestingly, α^+

thalassaemia also protected against hospital admission in children with non-malarial illnesses.

I investigated the frequencies of α^+ -thalassaemia and SAO, and their relationship with malaria in pregnancy and pregnancy outcome, in over 900 women who delivered their babies in hospital. My findings suggest that neither α^+ thalassaemia nor SAO protected against malaria in pregnancy or improved pregnancy outcome. During these studies, I identified the haematological and red-cell abnormalities most consistent with the band 3 deletion for SAO, which may be used to improve the diagnosis, where molecular methods are not available.

The findings of the potent protection afforded by α^+-thalassaemia and SAO against severe malaria in children will encourage further investigation of the underlying mechanisms. In turn, this may offer new approaches to the design of targeted interventions for the prevention and treatment of malaria that will improve the health of the millions of children who suffer from this devastating disease every year.

Chapter 1 Study background

1.1 Introduction

Between 1993 and 2006, I was responsible for the laboratory investigation of common red-cell polymorphisms and their interaction with malaria in children and pregnant women from the North coast of Papua New Guinea. This led to a series of publications, which form the basis of this application for a PhD by published works.

The aim of this programme of research was to investigate the relationship between malaria and those red-cell variants that are common in the North coastal region of Papua New Guinea (α- thalassaemia and Southeast Asian ovalocytosis [SAO]). This work was based on the hypothesis proposed by JBS Haldane in 1949, that the high frequency of the thalassaemias might reflect a heterozygote survival advantage against malaria. The clinical and laboratory features of malaria in children, and those features associated with mortality were described. I determined the frequencies of α- thalassaemia and SAO in children with malaria living in the community and those attending health facilities. I investigated the possible protection against severe manifestations of malaria and other severe illnesses in a case-control study of children admitted to hospital. I also investigated the effects of α- thalassaemia, SAO and malaria on pregnancy outcome. Finally, I described the haematological phenotype for SAO.

This research has provided a better understanding of the interaction between malaria and red-cell polymorphisms in children and pregnant women in PNG. This new information will facilitate the design of targeted interventions against malaria, to improve health in this region.

1.2 Malaria

Malaria is a major public health problem in tropical climates and is endemic in 91 countries in Africa, Asia and Latin America. Approximately 40% of the world's population live in malaria endemic areas (Hay *et al*, 2004). Children under the age of 5 years, pregnant women and non-immune travellers are particularly susceptible (World malaria report, 2005).

Each year there are 400-500 million infections and 1.2 -2.7 million deaths due to malaria (Webster & Hill, 2003). The majority (80%) occur in sub-Saharan Africa, where 10-30 % of all hospital admissions and 5-25% of deaths in children under the age of 5 years are attributable to malaria (World malaria report, 2005). During pregnancy, particularly the first pregnancy, women who previously had some immunity to malaria, lose this immunity and become highly susceptible to malaria, with grave consequences. Malaria in pregnancy is a major cause of severe maternal anaemia (haemoglobin level <7 g/dl), miscarriage, stillbirth, pre-term delivery, low birthweight and infant anaemia (Greenwood *et al*, 2005; Roll Back Malaria / WHO malaria in pregnancy guidelines, 2006).

Malaria is caused by four species of the unicellular protozoan parasite Plasmodium; *P. falciparum, P. vivax, P. ovale and P. malariae*. *P. falciparum* is the most important species, causing 50% of all malaria cases worldwide and responsible for severe malaria and nearly all deaths due to malaria (Greenwood *et al*, 2005). It is transmitted by the female Anopheles mosquito which breeds in hot, humid areas < 2000m. In the parasite's life cycle, a female anopheline mosquito takes a blood meal and injects sporozoites into the host. These very quickly migrate to the liver and undergo development within hepatocytes –this is termed the 'tissue' or 'exo-erythrocytic stage'. After about seven days the parasites are released from the liver into the blood stream and invade red blood cells where they multiply, consume host-cell nutrients and cause the red blood cells to rupture at frequent intervals – termed the 'erythrocytic' stage. It is the erythrocytic stage that is associated with fever. The parasites re-invade further red blood cells and cause further damage. Some parasites undergo a period of sexual development into male and female gametocytes and these forms are taken up by a mosquito when it takes a further blood meal. Gametocytes undergo a period of further development and fertilization within the mosquito to form oocysts within the insect gut wall. The oocysts contain thousands of sporozoites. After about ten days, the oocyst ruptures and the sporozoites migrate to the salivary glands and are injected into the host when the mosquito takes a blood meal, and so the cycle continues (Bell, 1994).

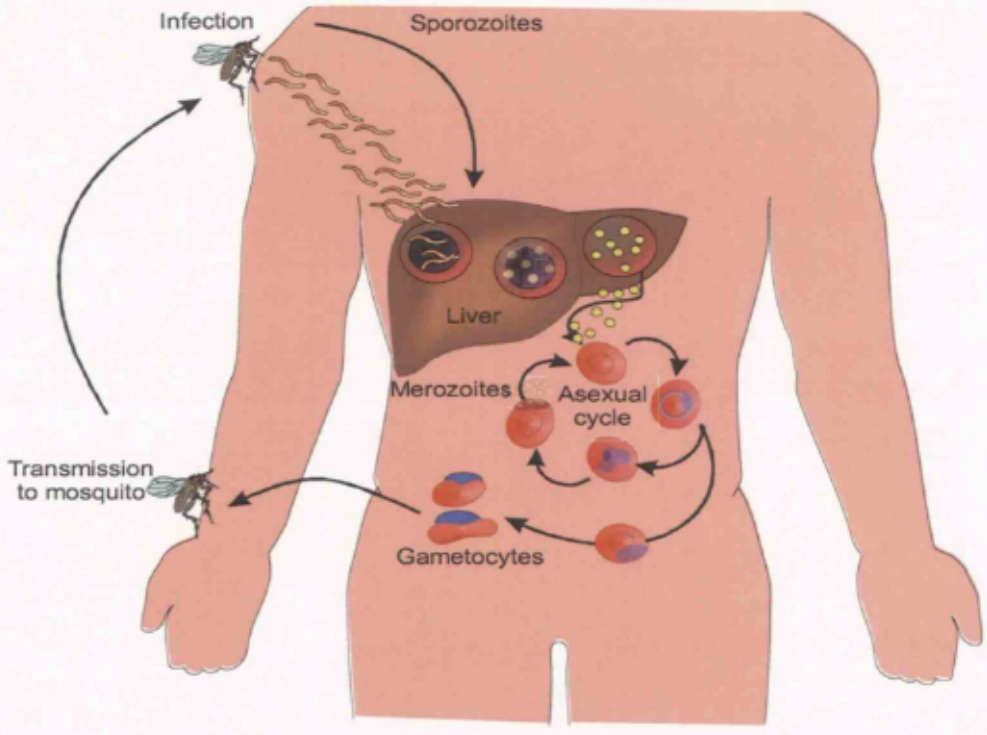


Figure 2: The life cycle of *P. falciparum*.

(Source: www.medicine.swan.ac.uk/documents/Malaria.ppt)

Sporozoites are injected into the human host when an infected female anopheline mosquito takes a blood meal. The sporozoites rapidly migrate to the liver where they undergo a period of development within the hepatocytes to form pre-erythrocytic schizonts, which contain thousands of merozoites. After about 7 days, the pre-erythrocytic schizonts rupture and merozoites are released into the blood stream where they enter the red cells. Following a period of asexual multiplication within the red cell, schizonts are formed, and after about 2-3 days the schizont and the red-cell rupture to release merozoites into the circulation. The merozoites invade new red cells and undergo a further period of asexual multiplication, and so the process continues. Some merozoites do not undergo asexual multiplication inside the red cells, but instead develop into male and female gametocytes which are infective to the female anopheline mosquito when it takes a blood meal. Within the mosquito gut wall, the gametocytes undergo a period of further development and fertilization to form oocysts, which contain thousands of sporozoites. After about ten days, the oocysts rupture and the sporozoites migrate to the salivary glands and are injected into the host when the mosquito takes a blood meal (Bell, 1994).

The clinical presentation of malaria consists of regular bouts of high fever and uncontrollable shivering, corresponding to when parasitized red cells rupture and release their contents. If treatment is not sought promptly, severe complications of malaria may ensue. The severe manifestations of malaria have been well documented in African populations (Warrell *et al,* 1990; Weatherall *et al,* 2002; Maitland & Marsh 2004). Briefly, the breakdown and destruction of red blood cells causes a massive loss of haemoglobin, which can lead to severe anaemia. Other severe complications of malaria include coma (where parasitized erythrocytes adhere to, and block, the small blood vessels that supply the brain), electrolyte inbalance, metabolic acidosis (low plasma bicarbonate and /or high plasma lactate) and hypoglycaemia (low blood sugar). Renal failure can also occur, particularly in adults. The urine may become dark in appearance; this is thought to be due to the release of haemoglobin from damaged red blood cells, which pass through the kidneys into the urine.

Malaria is usually diagnosed by the microscopic examination of Giemsa-stained thick and thin blood films. Other diagnostic tests include immunochromatographic strip tests to detect parasite antigens and the polymerase chain reaction (PCR) for the detection of parasite DNA, but their cost is prohibitive in the developing world.

Treatment of malaria needs to be both supportive and specific. Specific treatment is guided by national policy and documented drug resistance in the affected

country. In many parts of the world there is such wide-spread drug resistance that the cheaper antimalarials such as chloroquine and fansidar are no longer effective. Newer, more expensive combination drugs such as artemether + lumefantrine, artesunate + mefloquine, chlorproguanil + dapsone (Lapdap), atovaquone + proguanil (Malarone) are advocated, in the hope that the spread of drug resistance will be impeded. Since the drugs used in combination therapy each have a separate mode of action, the chance of selecting for resistant parasite strains will be reduced (Greenwood *et al*, 2005).

1.3 Thalassaemia

The thalassaemias are a heterogeneous group of blood disorders that result from the reduced or absent synthesis of α and β globin chains which make up normal adult haemoglobin ($\alpha_2 \beta_2$).

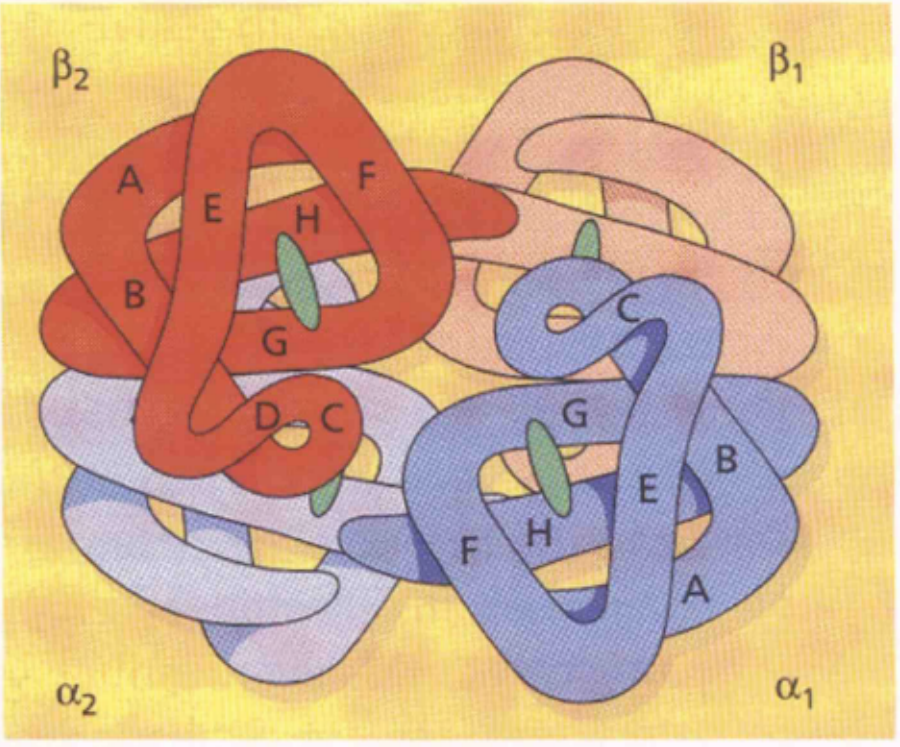


Figure 3: The quarternary structure of the haemoglobin A molecule.

Haemoglobin A is made up of 2 α and 2 β globin chains. Each chain contains a haem protein, which is essential for oxygen transport through-out the body (Bain, 2001a). Haem molecules are represented by green oval shapes. α-globin chains are represented in light/dark blue and β-globin in red/pink. Letters A-H represent the folds in the globin chains. Figure reproduced with the kind permission of Professor Bain and Blackwell Publishing Ltd.

Thalassaemia is found in many regions of the world, spanning from the Mediterranean basin, parts of Africa, throughout the Middle East, the Indian subcontinent, Southeast Asia, Melanesia and the Pacific (Weatherall & Clegg, 2001a). It reaches its highest frequencies in those parts of the world with the least resources to manage chronic ill health. Its distribution closely resembles that of malaria (past and present), with only a few notable exceptions; including

the high frequencies of thalassaemia observed in the "malaria-free" islands of Polynesia (Flint 1993; Weatherall & Clegg, 2001a).

1.4 α-thalassaemia

α- thalassaemia is the most common single gene disorder of humans. The genetics of α-thalassaemia is more complex than that of β-thalassaemia. There are two α-globin genes on each chromosome 16, and deletion of a single α gene results in α^+-thalassaemia.

There are eight recognised deletions that cause α^+-thalassaemia and these occur in either the α_1 or α_2 gene or as a result of a fusion α gene which is formed by unequal cross-over between α_1 and α_2 genes during meiosis (Bain, 2001c).

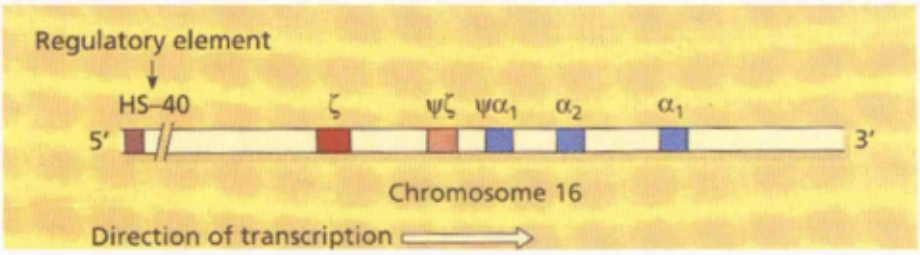


Figure 4: Arrangement of the α-globin gene cluster on chromosome 16 (Bain, 2001b).Expression of the structural genes is controlled by the upstream regulatory element –HS-40 which is situated 40 kilo bases upstream of the α_2 locus . The genes are arranged along the chromosome in the order that they are expressed during development. The ζ-globin gene is transcribed during early embryonic life and is then followed by a switch to α-globin gene production in early gestation which persists in fetal and adult life. Figure reproduced with the kind permission of Professor Bain and Blackwell Publishing Ltd.

α^+-thalassaemia, homozygotes have two α-globin genes (α -/α -) and heterozygotes have three α-globin genes (α -/ α α). Unlike β-thalassaemia, the

clinical effects of α^+-thalassaemia are mild; homozygotes have a mild hypochromic anaemia with haemoglobin levels about 1-2 g/dl lower than normals (normal haemoglobin concentration is in the range 11-14 g/dl), whilst red-cell indices in heterozygotes are often indistinguishable from normals. Deletion of both α-globin genes on a single chromosome results in α^0- thalassaemia. There are more than 20 recognised forms α^0- thalassaemia (Bain, 2001c). Homozygotes have no α-globin genes (--/--). This condition is lethal *in utero* and results in *hydrops foetalis.* Haematological indices in heterozygotes ($\alpha\ \alpha$ /--) are indistinguishable from homozygotes of α^+-thalassaemia (-α /- α) which means that the loss of two α-globin genes has the same effect on phenotype regardless of whether they are missing from the same or opposite pairs of chromosomes. α^0- thalassaemia also occurs when deletions in the upstream regulatory element HS-40 are found which inactivate the linked α-globin genes on the chromosome. Non-deletional forms of α^+-thalassaemia, caused by mutations that reduce the output of either α-globin gene also occur, but they are less common (Higgs, 1989; Higgs *et al*, 1993).

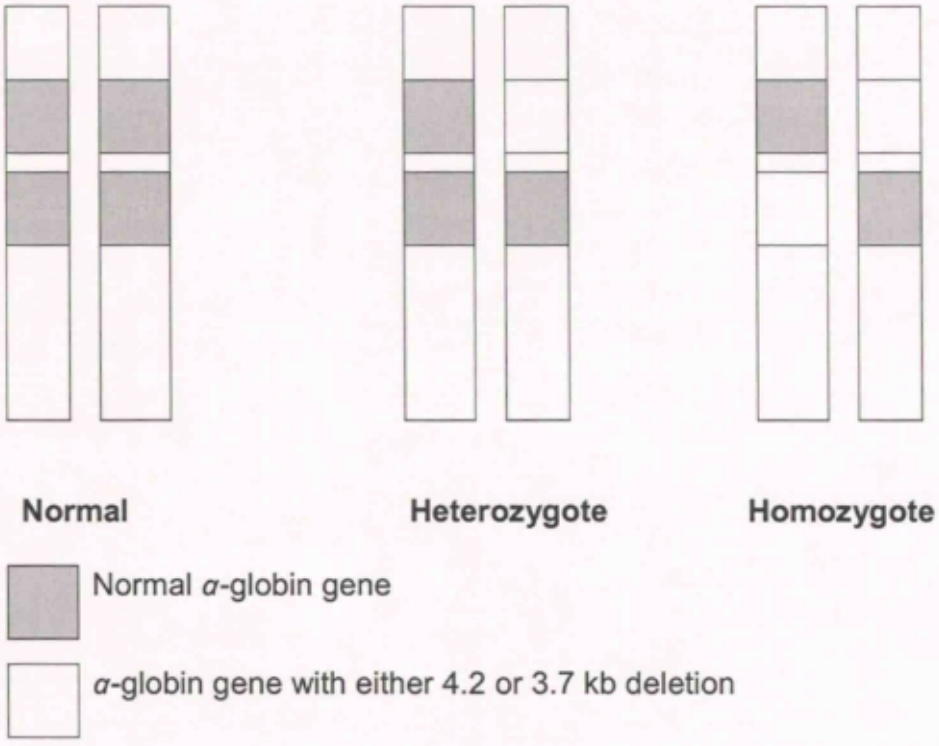
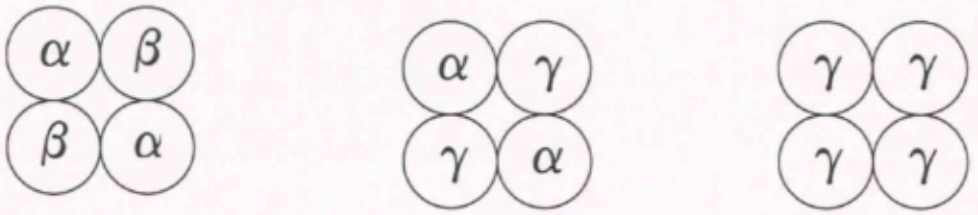


Figure 5: Schematic diagram of chromosome 16 showing common α-globin genotypes found in Papua New Guinea.

During the neonatal period, α-thalassaemia is characterized by increased levels of haemoglobin Bart's (Hb Bart's). Fetal haemoglobin (HbF), is the major haemoglobin in the neonatal period and is made up of 2 α-globin and 2 γ-globin chains. However, in α-thalassaemia, the deficiency of α-chain production, leads to an excess of γ chains, which form tetramers; Hb Bart's which is unstable and physiologically useless. Hb Bart's levels of 0.5 -1.0% are found in neonates with a normal α-globin genotype and detection of raised levels of Hb Bart's in neonatal blood samples, by electrophoresis, occurs in α^{+}-thalassaemia

homozygotes and α^0- thalassaemia heterozygotes, with Hb Bart's levels reaching 4-10%. (Weatherall & Clegg, 2001a; Weatherall & Clegg, 2001b). Haemoglobin electrophoresis is less useful in identifying α^+-thalassaemia heterozygotes, since elevated levels of Hb Bart's (1-2%) are found in some, but not all, neonates with this genotype (Weatherall & Clegg, 2001a).



Hb A ($\alpha_2\beta_2$) **Hb F ($\alpha_2\gamma_2$)** **Hb Bart's (γ_4)**

Figure 6: Schematic diagram of haemoglobin A, haemoglobin F and haemoglobin Bart's.

1.5 β-thalassaemia

There are two main types of β-thalassaemia; β^o-thalassaemia in which there is no β-globin production and β^+-thalassaemia which results in reduced β-globin synthesis. Over 200 β-thalassaemia mutations have been identified within the β-globin gene and flanking sequences on chromosome 11.

Many of these mutations are extremely rare, and most β-thalassaemia (80%) is due to about 20 common mutations, each with its own geographical specificity (Weatherall & Clegg, 2001a).

β-thalassaemia encompasses a wide spectrum of disease severity, ranging from the carrier state, classified as β-thalassaemia minor, to the blood- transfusion dependent state of β-thalassaemia major. β-thalassaemia minor is usually asymptomatic, with a slightly reduced haemoglobin concentration, increased red-cell count and reduced mean cell haemoglobin. β-thalassaemia major is characterized by severe anaemia, bone deformity, stunted growth, wasting of limbs, hepatomegaly and splenomegaly. A further group of diverse disorders which lie between these extremes are the β-thalassaemia intermedias which are characterized by anaemia, splenomegaly, bone deformity and leg ulcers; the severity of disease varying enormously between individuals (Rund & Rachmilewitz, 2005; Weatherall & Clegg, 2001c). Thus the clinical manifestations of β-thalassaemia are more complex and severe than for α^+-thalassaemia.

1.6 The malaria hypothesis

In 1949, JBS Haldane proposed that the high gene frequency of thalassaemia observed in Mediterranean populations was a result of natural selection for carriers of the thalassaemia gene. He proposed that the smaller, paler, more lysis-resistant red blood cells found in carriers of thalassaemia may also be more resistant to attack by malaria parasites. This innovative concept became known as the "malaria hypothesis" (Haldane, 1949).

To test this hypothesis, epidemiological studies to determine the geographical distribution of thalassaemia and malaria, laboratory investigations including *in vitro* studies to investigate parasite invasion into thalassaemic erythrocytes, the biological and immunological properties of thalassaemic cells and clinical case-control studies to investigate protection against malarial disease are all necessary. Population surveys undertaken in Italy in the late 1950's and early 1960's showed that there was good positive geographical correlation between the distribution of thalassaemia and malaria. Similarly, in Sardinia, a strong positive association between β -thalassaemia and malaria was observed. However, in studies in Cyprus, Greece, Malta, Sudan, New Guinea and Thailand, results were inconsistent (reviewed by Weatherall & Clegg, 2001a). A retrospective analysis of records from a malaria control programme in a malaria holo-endemic region of Southern Nepal, reported that the high frequency of α^+-thalassaemia in the Tharu native population accounted for a markedly decreased incidence of malaria. This was in comparison to the high incidence of malaria reported amongst immigrants to the area with no previous exposure to malaria and in whom the frequency of α^+- thalassaemia was <10% (Modiano *et al,* 1991).

In vitro studies have shown that parasite invasion is reduced in the severe forms of thalassaemia, such as HbH disease and homozygous HbE (Bunyaratvej *et al,* 1992), but is normal, over a single red-cell cycle, in erythrocytes from heterozygotes for α^+ thalassaemia and β- thalassaemia (Bunyaratvej *et al,* 1992; reviewed by Weatherall & Clegg, 2001a).

In vitro studies have also shown that *P. falciparum*- infected heterozygous red cells have reduced rosetting (a phenomenon whereby infected red cells bind uninfected red cells to form rosettes, which can cause obstruction in the blood vessels and is often associated with poor outcome) (Carlson *et al,* 1994). Binding studies have shown that thalassaemic red cells infected with *P. falciparum* bind more antibody per cell than normal red cells when treated with malaria hyperimmune serum (Luzzi *et al*, 1991).

Prior to my research, case-control studies to investigate the association between α-thalassaemia and clinical malaria had not been reported, although in a small prospective cohort study of Gambian children, α^{+}-thalassaemia was not shown to protect against asymptomatic parasitaemia or clinical episodes of malaria (Allen *et al*, 1993).

Haldane's hypothesis was not only applicable to thalassaemia but was also extended to include other haemoglobinopathies including sickle-cell anaemia. Between 1954 and 1971, numerous population studies to investigate the incidence of sickle-cell trait in malaria-endemic regions were conducted and there was growing evidence that sickle-cell heterozygotes were protected against severe malaria infection (Allison, 1954; reviewed by Weatherall & Clegg, 2001a). More recently, this has been shown conclusively in studies conducted in The Gambia, where sickle-cell heterozygotes have a 10-fold reduced risk of severe malaria (Hill *et al,* 1991; Ackermann *et al,* 2005).

Epidemiological and some case-control studies to investigate the incidence of other common haemoglobin and red-cell variants such as haemoglobins C and E, Duffy blood group antigen and glucose-6-phosphate dehydrogenase (G6PD) deficiency have shown that they too reach high frequencies in malarious regions. It is particularly interesting to note that they each have restricted geographic distributions suggesting that different haemoglobin and red-cell variants have evolved in different populations in response to malaria. For example, the HbS allele is common in West Africa, with frequencies of 15 to 30% reported in Ghana and Nigeria (WHO, 2006), but is almost absent in South East Asia. Whereas, the HbE allele occurs in the eastern parts of the Indian sub-continent, Burma and South east Asia, with frequencies of up to 60% reported in some parts of Thailand, Laos and Cambodia, but is absent in the African continent (Vichinsky, 2005). Similarly, the absence of the Duffy blood group antigen, which ensures complete resistance to *P. vivax* infection, occurs almost exclusively in sub-Saharan Africa.

The distribution of haemoglobin variants may also differ at the local level. For example, in the Dogon population of Mali, West Africa, the frequency of the HbS allele is 3% and is much lower than in other countries in this region, but the frequency of the HbC allele is 16% and is higher than in other West African countries (Agarwal *et al*, 2000). All of the above-mentioned haemoglobin and red-cell variants have been shown to either confer protection against clinical

malaria or, *in vitro* studies suggest that affected erythrocytes are resistant to invasion by *P. falciparum*. In a study of 4,348 subjects from Burkina Faso, West Africa, a 29% and 76% reduction in the risk of clinical malaria was found in HbC heterozygotes and homozygotes respectively (Modiano *et al*, 2001). In a study in Thailand, *in vitro* invasion studies of *P. falciparum* into HbE heterozygous cells was reduced to 25% compared to invasion into erythrocytes with normal haemoglobin (Chotivanich *et al*, 2002). G6PD deficiency is X-linked and is the most common red-cell enzymopathy in humans. In studies of over 2000 African children, it has been shown to be associated with a 46-58% reduction in the risk of severe malaria in heterozygous females and hemizygous males (Ruwende *et al*, 1995).

Chapter 2 Papua New Guinea

2.1 Papua New Guinea

The Independent State of Papua New Guinea (PNG) is a country in the Southwest Pacific ocean of Melanesia, which gained independence from Australia in 1975. It comprises the eastern half of the island of New Guinea (the western side is Indonesia) and the many offshore islands of the Bismark Archipelago and occupies a total surface area of 463 Km^2. The capital is Port Moresby, situated on the South coast of the island.

Figure 7: Map of Papua New Guinea.

(Source: Central Intelligence Agency world fact book –www.cia.gov/library/publications/the-world-factbook/geos/pp.html)

The geography of the country is extremely diverse and extends from the hot, tropical islands and lowlands to the rainforests and on to the cooler, steep, rugged, highlands. Annual rainfall varies from 1300 to 7000 mm, with some rain throughout the year (Mueller, 2003). The population of PNG is almost 6 million and is both culturally and linguistically diverse, with more than 800 different tribal groups or clans and over 850 different languages spoken throughout the country (Reeder, 2003). Almost 80% of the population live in rural areas and rely on the subsistence farming of bananas, cocoa, coconut, coffee, palm kernels, sweet potatoes and yams for their livelihood. The land is rich in natural minerals and resources and the economy relies heavily on exports of copper, gold, logs and oil (Central Intelligence Agency world fact book).

PNG has the worst health statistics in the South Pacific (Reeder, 2003). The average life expectancy at birth is 59 years for males and 63 years for females (WHO, 2004). Infant mortality rates are 61/1000 livebirths for females and 67/1000 livebirths for males, under five-year mortality rate is 99/1000 livebirths and maternal mortality rate is 300/100,000 (WHO, 2004). Malaria and pneumonia are the most common causes of illness and death (Reeder, 2003). The country is facing an HIV/AIDS epidemic, and other major health problems include malnutrition, tuberculosis and diabetes.

2.2 Malaria in PNG

Malaria poses a huge problem to human health in PNG, with more than 25% of out-patient attendances and 12% of all deaths being due to malaria infections (Genton, 2003). Malaria occurs mainly in the coastal and lowland areas below 2500m, where approximately two-thirds of the population lives. All four *Plasmodium* species that infect humans occur in PNG (*P. falciparum, P. vivax, P. malariae* and *P. ovale*). *P. falciparum* is the predominant species throughout the country and is responsible for most of the morbidity and mortatility due to malaria. However, in areas of very low endemicity, *P. vivax* predominates. *P. malariae* occurs less frequently and *P. ovale* is very rarely found (Mueller, 2003). The highland areas are reportedly "malaria free" (Hill, 1988), although there are recent reports of outbreaks in the highland fringes (Mueller, 2003). Malaria transmission is hyperendemic in Madang Province, situated 5° south of the equator on the North coast of PNG. Surveys undertaken in 72 villages from this region reported that *P. falciparum* is transmitted throughout the year and parasite rates were greatest in children aged 5-9 years (Cattani *et al*, 1986). Mortality surveillance conducted in 35 villages in the same region, during the early 1980's determined that at least 11% deaths in children under 10 years old were attributable to malaria (Moir & Garner, 1986).

Studies of malaria in pregnancy carried out by Brabin *et al* (1990b) in Madang Province, showed that susceptibility to *P. falciparum* malaria is increased in primigravidae (women in their first pregnancy). Severe malaria anaemia was

common during pregnancy, occurring in 44% of primigravidae and 29% multigravidae (women in their second or more pregnancy) and was associated with low birthweight but not pre-term delivery, in primigravidae (Brabin *et al,* 1990a). Finally, in a study of more than 12,000 singleton births from both Madang and the non-malarious highlands to assess the individual impact of anaemia or malaria on low birthweight, striking differences were reported; 89% of pregnant women from Madang were anaemic compared to 29% in highlands, and the Madang women were three times more likely to have a low birthweight baby than pregnant women from the highlands. Malaria may account for up to 40% of low birthweight babies and up to 10% of severe anaemia in babies born in malarious areas (Brabin & Piper, 1997).

2.3 Haemoglobin and red-cell polymorphisms found in PNG

A number of haemoglobin and red-cell polymorphisms occur in PNG, particularly in the malaria coastal regions, and include HbJ Tongariki, Gerbich-negative blood group, G6PD deficiency, Southeast Asian ovalocytosis (SAO), and both α and β-thalassaemia.

2.4 HbJ Tongariki

This abnormal haemoglobin was discovered in 1967 in Tongariki island in the New Hebrides, Melanesia (Gajdusek *et al*, 1967). It is associated with α-thalassaemia and is due to a base change at codon 115 of the α-chain locus on

chromosome 16, in which the partner α-globin gene has been deleted. It has a limited distribution in PNG, and is found mainly on Karkar Island (situated about 15 Km off the coast of Madang) at low frequency; 4% (Beavan *et al,* 1972). A study on Karkar Island suggested that HbJ Tongariki may increase susceptibility to *P. vivax* infection (Harrison & Boyce, 1976).

2.5 Gerbich – negativity

Gerbich negativity is due to a deletion of exon 3 in the glycophorin C gene on chromosome 2. Glycophorin C is a receptor on the erythrocyte surface which interacts with the *P. falciparum* erythrocyte binding antigen 140 (EBA 140) to facilitate parasite invasion into the erythrocyte. Positivity for the Gerbich blood group antigen is almost universal. However, in some coastal areas of PNG, Gerbich negativity reaches frequencies of up to 47% (Patel *et al*, 2001). Epidemiological data suggests that Gerbich negativity is associated with reduced malarial infection (Serjeantson, 1989), and *in vitro* studies have recently shown that *P. falciparum* is unable to invade Gerbich negative erythrocytes (Maier *et al*, 2003).

2.6 Red blood cell complement receptor 1 (CR1)

Complement receptor 1 is expressed on the surface of red blood cells. It is the receptor which mediates the adhesion of parasitised to non-parasitised red blood cells to form clumps or "rosettes" *in vitro*. This process of "rosetting" is thought to contribute to the pathogenesis of severe malaria. (Cockburn *et al*, 2004). Red

blood cells deficient in CR1 show greatly reduced rosetting. In PNG, CR1-deficiency occurs in up to 80% of the population and is associated with a polymorphism in exon 22 of the CR1 gene. This polymorphism has been shown to be associated with protection against severe malaria in children in PNG (Cockburn *et al*, 2004).

2.7 G6PD deficiency

G6PD regulates the reduction of NADP to NADPH within erythrocytes. NADPH is important because it protects the red-cell from oxidant damage. The intra-erythrocytic stages of *P. falciparum* are sensitive to oxidant stress and are therefore disadvantaged in G6PD deficient cells. More than 22 different forms of G6PD deficiency have been characterized in PNG, with an overall prevalence estimated at 6.7% (Yenchitsomanus *et al*, 1986). G6PD deficiency is absent in the highlands, but frequencies of up to 53% have been reported in the eastern Morobe province of the Island (Serjeantson, 1992). In contrast to its protection against malaria, G6PD deficiency can cause haemolytic anaemia in individuals exposed to oxidizing drugs such as primaquine or following the consumption of certain foods, fava beans for example.

2.8 Southeast Asian Ovalocytosis (SAO)

The molecular basis for SAO is a 27 base-pair deletion in the boundary between the cytoplasmic and first transmembrane domain in the gene encoding band 3, the major erythrocyte transmembrane protein, on chromosome 17. In the

heterozygous state SAO is asymptomatic but the homozygous state is thought to be lethal *in utero* (Liu *et al*, 1994). SAO is characterized by alterations in red blood cell appearance and anion transport is reduced to about 40% of normal (Schofield *et al*, 1992). Prior to the molecular identification of SAO, epidemiological studies to determine the frequency of SAO in PNG relied on examination of thin blood films for the presence and proportion of oval shaped red cells (Serjeantson *et al,* 1977; Cattani *et al,* 1987). SAO occurs commonly in the malarious regions of Southeast Asia and the western Pacific; frequencies of 35% have been reported for people living in the North coast of PNG (Mgone *et al*, 1996). Interestingly, SAO is not found in the highly malarious Sepik region of PNG. In two studies carried out in the malarious North coast of PNG, Serjeantson *et al* (1977) reported no association between malaria parasite density and SAO, but Cattani *et al* (1987) found a reduced frequency of malaria in children with >50% ovalocytes present in thin blood films. With the advent of DNA technology, SAO diagnosis became more accurate and in a small clinical study in the North coast of PNG, SAO was found to protect against cerebral malaria (Genton *et al,* 1995). However, in this analysis, other factors that reduce malarial disease were not taken into account.

2.9 β-thalassaemia

In PNG, β-thalassaemia is more prevalent in the coastal and lowland malarious areas of Milne Bay, Morobe and Oro provinces than in the highland areas where it's occurs sporadically (Serjeantson, 1992). In the highly malarious region of

Madang Province, β-thalassaemia frequencies of about 1.5% have been reported (Serjeantson, 1992).

Since β-thalassaemia has more severe clinical implications (such as a requirement for regular blood transfusions) than α-thalassaemia, it is proposed that the lower frequency of β-thalassaemia occurs as a result of more effective selection for the relatively benign α-thalassaemia (Serjeantson, 1992).

2.10 α-thalassaemia

Before the advent of DNA technology, epidemiological studies describing the geographical distribution of α- thalassaemia were few, and were based solely on the measurement of Hb Bart's levels in cord blood samples. This may have led to an underestimation of the true frequency of α^+- thalassaemia in some populations; Hb Bart's may not be detected in the neonatal period in some infants with a single α-globin gene deletion (ie. α^+-thalassaemia heterozygotes). Hence it is not surprising that there was little evidence to suggest that the frequency of α- thalassaemia correlated with malaria. By the mid 1980's, studies of the distribution of α- thalassaemia in the South West Pacific (Melanesia) and its relationship to malaria were undertaken using DNA analysis (Flint *et al,* 1986; Yenchitsomanus *et al,* 1986) and there was a striking latitude and altitude correlation between α^+-thalassaemia gene frequencies and malaria endemicity (Flint *et al*, 1986). A latitudinal cline of frequencies of α^+- thalassaemia through

Melanesia was observed with frequencies of up to 70% in the North coast of PNG falling to less than 10% in New Caledonia in the South east of Melanesia. Similarly, marked differences in the prevalence of α^+- thalassaemia were observed with altitude within PNG. Frequencies of 4% were observed in the highlands (above 1500m) whereas frequencies of 39% were found in the lowland coastal areas. The highest frequencies of α-thalassaemia in the world are found in the coastal areas of PNG.

PNG provides a unique opportunity to investigate the association of α^+-thalassaemia and malaria. Since abnormal haemoglobin variants such as HbS, HbC and HbE are not found in PNG and β-thalassaemia is uncommon, these associations are easier to interpret, as there are very few confounding effects due to other haemoglobinopathies. This is not the case in Africa or the Indian sub continent.

Chapter 3 Programme of work undertaken by the applicant

The research was initiated by Professor Sir David Weatherall and Professor John Clegg, Weatherall Institute of Molecular Medicine (WIMM), Oxford, and was funded by the Wellcome Trust and Medical Research Council. The other main member of the research team was Dr. Stephen Allen who undertook the clinical work of the project.

My involvement in this research began in June and July 1993 when I was based in Oxford. I contributed to the design of the studies, as part of the research team. These discussions led to the conduct of a case-control study in children and a cohort study of pregnant women. It was clear that in addition to DNA extraction and analysis, a wide range of laboratory investigations would be required to complement the clinical assessments. I advised on the range of laboratory instruments and procedures that would be appropriate for PNG and required for the identification of anaemia, haemoglobinopathies, the severe manifestations of malaria and other common causes of an acute febrile illness such as meningitis and urinary tract infection. I trialed all laboratory procedures in Oxford. I also undertook training in instrument maintenance and basic repairs for the haematology and biochemistry analyzers and established means of accessing remote advice in preparation for operating these instruments in the relatively isolated situation of PNG.

In August 1993, I moved to Madang, Papua New Guinea and was primarily responsible for the development and execution of the laboratory studies of this research. I identified a vacant science teaching laboratory in the College of Allied Health Sciences that was close to Madang hospital and could serve as both the project laboratory, as well as the main project office.

Figure 8: College of Allied Sciences Laboratory.

I converted this laboratory into a multi-disciplinary research laboratory equipped with instruments and analyzers that I had sourced from numerous suppliers and arranged to be shipped from Oxford in the previous months. I set-up a wide range of laboratory investigations for this programme of research and these included:

i. Measurement of biochemical indices such as plasma sodium, potassium, bicarbonate, lactate, glucose, creatinine, bilirubin, liver enzymes and cardiac enzymes by dry slide chemistry, using the Kodak Ektachem DTE, DT60 and DTSC analyzers.

ii. Measurement of haematological indices using a Coulter MD8 instrument.

iii. Preparation and screening of red-cell lysates for the presence of abnormal haemoglobin variants by cellulose acetate electrophoresis.

iv. Screening tests todetermine G6PD deficiency using a visual semi-quantitative assay (Sigma procedure no. 400).

v. Measurement of G6PD enzyme activity and plasma haemoglobin using commercially available colorimetric tests (Sigma procedure nos. 345-UV and 527, respectively).

vi. Preparation and Giemsa staining of thick and thin blood films for the presence of malaria parasites and determination of red-cell morphology by microscopy.

vii. Reticulocyte determination by identification of reticulocyte ribosomal RNA in thin blood films prepared with New Methylene blue stain.

viii. Determination of red-cell osmotic fragility by suspending red blood cells in saline solutions of decreasing concentration and colorimetrically determining the concentration of saline at which 50% of the red-cells were lysed.

ix. Screening urine samples for the presence of protein, ketones, blood and haemoglobin using Combur-7 test strips (Boehringer Mannheim) and examination of urine sediment by microscopy.

x. Microscopic examination and measurement of white blood cells present in samples of cerebral spinal fluid (csf) using a Nebaurer counting chamber.

xi. Screening csf samples for meningitis using a latex agglutination assay (Biomerieux) and measurement of csf glucose and protein levels by dry slide chemistry and colorimetry, respectively.

xii. Preparation of DNA samples from blood cell pellets by proteinase-k digestion and phenol-chloroform extraction.

To assist in the laboratory work, I recruited Martha Mellombo, a local school leaver with no previous laboratory experience. I trained her in basic laboratory techniques including sample labeling, record keeping, blood film preparation and staining, and operation of the Coulter haematology analyzer. I also negotiated additional assistance with DNA extraction from the PNG Institute of Medical Research laboratory staff.

Figure 9: Laboratory before and after renovation and installation of instruments.

Between October 1993 and February 2006, I was responsible for the analysis of over 3000 samples of blood, urine and cerebral spinal fluid from children and pregnant women from Madang PNG. I ensured reliability of results by implementing internal and external quality control procedures for all assays,

including NEQAS for biochemical measurements and haematology controls shipped monthly from Coulter, Australia. I was responsible for the correct labeling, recording, storage and shipment of frozen samples of serum, plasma, DNA, urine and cerebral spinal fluid, to Oxford. I undertook further laboratory investigations on shipped DNA samples, to determine α-globin genotype by Southern blotting and hybridisation and the band 3 deletion for Southeast Asian ovalocytosis by pcr, following my return to Oxford in April 1996.

The results of the laboratory investigations that I performed in PNG formed a major part of the first publication of this research in the Quarterly Journal of Medicine in 1996, in which the clinical and laboratory features of severe malaria were described. I contributed to the writing of the manuscript. In particular, I performed a literature search and sourced the references and wrote the laboratory methods and results section of the paper.

During the course of my work in PNG, an interesting observation emerged which was the presence of dark urine in children with malaria. Upon returning to Oxford briefly, in 1994, I discussed this observation with both Professor Sir David Weatherall and Professor David Warrell, and they encouraged me to investigate this further. I identified and undertook the necessary laboratory investigations and arranged for the collection of repeat blood and urine samples from children in convalescence. I enlisted assistance from Dr Ann Taylor with measurements of myoglobin in plasma and urine in samples shipped to Oxford. I contributed to

the data entry and analysis, and wrote the first draft of the manuscript which was published in the Transactions of the Royal Society of Tropical Medicine and Hygiene in 2006.

After I had determined α-globin genotypes in DNA samples, and laboratory indices of severe malaria in children recruited in the case-control study, complex statistical analyses of α -globin genotype according to case-control status and manifestation of severe malaria amongst cases were undertaken by Drs. Neal Alexander and Tim Peto. I contributed to the interpretation of the findings and the writing of the manuscript. In particular, I did the literature search and obtained the relevant references, wrote the laboratory methods section of the manuscript and helped with the presentation of the results. This led to the **first** publication to report that α-thalassaemia provided potent protection against severe malaria in children in PNG and was published in PNAS, USA in 1997.

The focus of my research then turned to the band 3 deletion for SAO. During my work in Papua New Guinea, I had prepared and examined thin blood films from all study participants. After I had determined the band 3 SAO genotype in DNA samples, I undertook a detailed description of the red-cell morphology and haematological features that were characteristic of the SAO band 3 deletion. My findings were published in the British Journal of Haematology in 1998. In addition to designing this study and performing all of the laboratory investigations, I contributed to the data entry and analysis and wrote the first draft of this paper.

I then went on to investigate the possible interaction between the SAO band 3 deletion and malaria. I had previously determined the band 3 SAO genotype in DNA samples collected from children in the case-control study, and these results were verified by Dr Charles Mgone, who repeated the genotyping on a sub-set of the DNA samples. Complex statistical analyses of band 3 SAO genotype according to case-control status and manifestation of severe malaria amongst cases were undertaken by Drs. Neal Alexander and Tim Peto. This led to the publication of a paper in The American Journal of Tropical Medicine and Hygiene in 1999 in which the fascinating finding that the SAO band 3 deletion provided potent protection against cerebral malaria was reported. In addition to my contribution to the study design and its execution, I contributed to the data analysis, interpretation of the findings and writing of the manuscript. In particular, I performed the literature search and obtained the relevant references, wrote the laboratory methods section and presented the laboratory findings in the results section of the paper.

In 2003, I re-located to Swansea, where I have continued research into the pregnant women who delivered their babies at Madang Hospital between July 1994 and February 1996, and in whom I had assisted Dr Allen with an investigation of the causes of preterm delivery and intrauterine growth retardation (published in 1998 in the Archives of Diseases in Childhood).

The remarkable finding of protection against malaria and cerebral malaria in children afforded by α-thalassaemia and band 3 SAO deletion respectively, together with the fact that pregnant women, particularly those in their first pregnancy are more susceptible to malaria, prompted me to extend our previous studies by investigating the effect of these red-cell polymorphisms on pregnancy outcome and malaria during pregnancy. I determined α-globin genotype and band 3 SAO genotype in DNA samples that I had extracted from blood samples, collected from a cohort of more than 900 pregnant women, during my work in PNG.

I correlated α -globin genotype and SAO band 3 genotype with reproductive fitness, pregnancy outcome and malaria during pregnancy, using the SPSS statistical analysis software. I published my findings relating to α – globin genotype and pregnancy in the British Journal of Haematology in 2006. My findings relating to SAO band 3 genotype and pregnancy were published in the American Journal of Tropical Medicine and Hygiene in 2007.

In summary, I feel greatly privileged that this programme of research allowed me to develop skills in study design, data analysis and writing manuscripts for publication. I also gained extensive further experience in setting-up and under-taking a wide range of laboratory procedures and training and supervising laboratory staff in the challenging environment of a resource-poor country.

Chapter 4 Review of published works

4.1 Severe manifestations of malaria in children admitted to hospital

The clinical and laboratory features of severe malaria in children admitted to hospital were reported in the Quarterly Journal of Medicine in 1996 and the cause of dark urine in some of these children was reported in the Transactions of the Royal Society of Tropical Medicine and Hygiene in 2006 and are summarized below.

1. Allen SJ, **O'Donnell A**, Alexander NDE, Clegg JB. Severe malaria in children in Papua New Guinea. *Quarterly Journal of Medicine* 1996; 89: 779-788.

Mortality from malaria is a relatively uncommon event and very large studies would be required to use mortality as an endpoint. Therefore, studies that assess the survival advantage of a haemoglobinopathy or other genetic trait against malaria usually use severe manifestations of malaria as a surrogate for mortality. The clinical features of malaria and the severe manifestations associated with case fatality had been described in African children. These studies enabled the WHO to define a limited number of clinical and laboratory features of malaria as severe manifestations. However, the clinical and laboratory features of malaria and the manifestations associated with case fatality had not been described in

detail in children in PNG. Therefore, this was an initial priority for this programme of research as a basis for the assessment of the protective effect of haemoglobin variants.

Between October 1993 and February 1996, the clinical and laboratory features of severe falciparum malaria and risk factors for mortality were investigated in 489 children who were admitted with malaria to Madang Hospital. Only children who had lived in Madang Province for at least one year were recruited. Demographic and clinical details were recorded on admission. A detailed medical examination was undertaken and signs of respiratory distress and shock were noted and the level of consciousness was assessed using the Blantyre coma score (Molyneux *et al*, 1989).

Figure 10: The children's ward at Madang District Hospital.

A venous blood sample was collected from each child and transferred promptly to the laboratory for analysis. A lumbar puncture was performed in all comatose children (with no contraindications) and a specimen of cerebrospinal fluid (CSF) was collected. A urine sample was also collected from each child. Laboratory investigations included measurement of routine haematological data, biochemical indices including plasma sodium, potassium, bicarbonate, lactate, glucose, creatinine, bilirubin and liver enzymes. Thick and thin blood films were prepared, stained with Giemsa and examined for the presence of malaria parasites. CSF was examined by microscopy and the glucose level measured. Urine samples were tested with Combur 7 test strips (Boehringer Mannheim, Germany).

All children were managed according to standard protocols and were reviewed at least twice a day. Following discharge, those children admitted with coma were visited in the community and examined for persisting neurological deficits.

Statistical analyses included the Mann-Whitney test for analysis of continuous laboratory and clinical variables and the χ^2 or Fisher's exact test for categorical variables. Associations between the severe manifestations of malaria were assessed using 2 x 2 tables and Pearson's correlation co-efficient was used to assess associations between continuous variables. P values < 0.05 were considered significant. Univariate logistic regression and multiple regression

analyses were used to determine risk factors for mortality. All analyses were performed using SPSS (version 7).

Complete data were available for 419/489 (86%) children admitted with malaria, of whom 220 (52%) had at least one severe manifestation. The most common severe manifestations were severe anaemia (Hb <5 g/dl; 22% of cases) and coma (Blantyre coma score ≤2; 16%). Hyperlactataemia (plasma lactate ≥5 mmol/l), metabolic acidosis (plasma bicarbonate <15 mmol/l), jaundice and respiratory distress occurred in 20%, 13%, 12% and 10% of children, respectively. Hypoglycaemia (plasma glucose < 2.2 mmol/l) and hyperparasitaemia (*P. falciparum* parasitaemia ≥500,000 / μl) were less common, occurring in 3% and 2% of children respectively. Renal failure (plasma creatinine > 265 μmol/l) occurred in one child and shock in two children.

Severe anaemia tended to occur in younger children (median age 2.2 years) who had been ill for a median of 7 days. In contrast, coma occurred in older children (median age 3.7 years) who had a shorter duration of illness (median 4 days). Generalized convulsions before admission were common in children with coma (85%) but also occurred in 35% children without severe disease. Anaemia was associated with hyperlactataemia and was strongly negatively associated with coma. Coma was associated with hypoglycaemia and also with hyperlactataemia, but was not associated with low plasma bicarbonate.

Seventeen (3.5%) children died in the study. Deaths were more common in older children and were greater in those of Sepik ethnicity who resided in the peri-

urban settlement areas of Madang. In multiple regression analysis, a high level of plasma lactate (≥5 mmol/l) was the major predictor of death. Raised plasma creatinine and decreased plasma bicarbonate were also independent predictors of mortality. The observation that high plasma lactate and low plasma bicarbonate were not associated suggests that these two markers of metabolic acidosis may have different causes. Surprisingly, haemoglobin had a positive association with mortality, indicating greater mortality at higher haemoglobin concentrations. Coma was not predictive of death, although 5/10 (50%) children with profound coma (Blantyre coma score of 0) died.

Upon inspection of urine samples, an unexpected finding was the occurrence of dark (brown or red) urine in over 20 samples. An investigation into the cause of dark urine was undertaken and reported (see below).

P. falciparum parasite density in the peripheral blood was greater in children who died than in those who survived and was positively associated with low plasma bicarbonate and hyperlactataemia. *P. falciparum* parasite density was similar in children with and without coma.

Longterm follow-up of 66 children with coma showed that 4 (6.1%) had persisting neurological deficit.

Prior to this study, clinical descriptions of malaria in children in Papua New Guinea were few (Darlow, 1981; Stace *et al*, 1982; Vince, 1992) and severe manifestations of malaria poorly defined. A retrospective analysis of records of 68 children admitted to Madang hospital with cerebral malaria in 1980 found that

children with cerebral malaria were significantly older than children with uncomplicated malaria and the mortality rate was 5.9%. (Stace *et al*,1982). Hypoglycaemia was identified as a major complication of severe malaria in children in PNG (Vince, 1992).

Following the publication of WHO criteria defining severe and complicated malaria, it was possible to compare the clinical features and risk factors for mortality in the PNG study cohort with similar studies from Malawi, Kenya and The Gambia (Molyneux *et al,* 1989; Newton *et al,* 1991; Waller *et al,* 1995). Many clinical features of malaria in the PNG cases were similar to those reported in these African studies. These included age, duration of illness, neurological features and the frequency of severe anaemia (Marsh *et al*, 1995; Modiano *et al*, 1995). High plasma lactate was the major risk factor for mortality in the PNG children and this is in keeping with studies in African children (Marsh *et al*, 1995; Taylor *et al*, 1993). However, there were several differences. A notable difference was the lower parasite density in children with coma (median value 3.83 $\log_{10}$ parasites /μl) and also the reduced frequency of neurological deficit (6.1%) in the PNG children compared to 10% reported in African studies (Molyneux *et al*, 1989; Brewster *et al*, 1990; Bondi, 1992). Also, despite the clinical features associated with coma in children with malaria from Madang resembling closely those reported in African children, mortality amongst Madang children with coma (8.0%) was markedly lower than that reported in African studies (range 15 - 24.7%) Molyneux *et al*, 1989; Newton *et al*, 1991; Waller *et al*, 1995; Modiano *et al*, 1995; Bondi, 1992),

4.2 Dark urine

2. **O'Donnell A**, Weatherall DJ, Taylor AM, Reeder JC, Allen SJ. Muscle- cell injury, haemolysis and dark urine in children with falciparum malaria in Papua New Guinea.*Transactions of the Royal Society of Tropical Medicine and Hygiene* 2006; 100: 817-25.

During the clinical study described above, an unexpected observation was that several children passed Coca-Cola coloured urine. This suggested a diagnosis of "blackwater fever" (BWF), that is the passage of dark brown or red-coloured urine thought to be due to haemoglobinuria as a result of severe acute malaria-induced haemolysis and often complicated by renal failure (Warrell *et al*, 1990). It is well documented in people with limited malaria immunity living in malarious areas (Findlay, 1949) and in indigenous adults (Dellacollette *et al* 1995; Tran *et al,* 1996) but rarely in children (Rogier *et al,* 2003). Glucose-6-phosphate dehydrogenase (G6PD) deficiency, unstable haemoglobin phenotypes such Hb Hammersmith and Hb Koln and quinine usage are all factors reported to contribute to haemolysis in BWF (Tran *et al*, 1996). However, in view of a report of dark urine attributable to myoglobinuria in a Nigerian male who had malaria complicated by rhabdomyolysis (Knochel & Moore, 1993) and since routine laboratory tests fail to distinguish between haemoglobin and myoglobin in urine, I undertook a comprehensive investigation to identify the cause of dark urine in the

PNG study children. Details of these children and clinical methods are described above in paper 1.

A urine sample was collected on admission from each child, examined immediately and tested with Combur 7 test strips for the presence of a haem protein. Urine samples were also collected from children with a primary illness other than malaria and tested as above. In addition to those laboratory methods detailed previously, further assays to investigate haemolysis and muscle-cell injury were undertaken in all children whose urine was dark in colour and contained a haem protein. These assays were also undertaken in two representative groups of control children from the study who had acute malaria but urine of normal appearance. One control group had urine that tested positive for a haem protein and the other group had normal urine.

In order to differentiate between haemoglobin and myoglobin in urine, samples were precipitated in 80% ammonium sulphate. To assess haemolysis, plasma haemoglobin and serum haptoglobin were measured. Giemsa stained thin blood films were examined for the presence of schistocytes and the Direct Coombs test (DCT) was undertaken to detect complement or antibodies on red cells (Dia Med, Midlothian, Scotland). Screening tests to detect G6PD deficiency and abnormal haemoglobin phenotypes were also undertaken. Laboratory methods to investigate muscle-cell injury included the measurement of plasma creatine kinase (CK) and its cardiac isoenzyme CKMB, and plasma and urine myoglobin.

Children who had passed red or brown urine were visited in convalescence, between 1 and 9 months after discharge to investigate for chronic haemolysis. Blood samples were collected for measurement of G6PD activity, serum haptoglobin, plasma bilirubin, full blood count, reticulocyte count and red-cell osmotic fragility. Urine was collected for testing with Combur 7 test strips for the presence of a haem protein and the detection of haemosiderin.

Statistical methods were as described previously.

A urine sample was collected from 351/489 (71.8%) children admitted to hospital with malaria. A haem protein was detected in 115 samples (32.8%) and of these, 22 urine samples were dark (Group I) and 93 were normal colour (Group II). 236 children had normal urine (Group III). The urine of 4/179 children admitted to hospital with a primary diagnosis other than malaria contained a haem protein and was dark in colour. The ammonium sulphate precipitation test to differentiate between haemoglobin and myoglobin in urine was difficult to interpret and was abandoned. There was greater evidence of intravascular haemolysis in Group I: plasma haemoglobin levels were greater, serum haptoglobin levels lower and schistocytes were more common than in Groups II and III. Plasma haemoglobin levels above the renal threshold for haemoglobinuria (30-60 mg/dl) (Weatherall, 1996; Ekvall *et al*, 2001) were observed in Group 1 children. However, a surprising finding was that anaemia was greater in Group III. Given the preceding laboratory results, this is likely to have resulted mainly from extravascular haemolysis. This is consistent with the findings in 20 Tanzanian children with malaria in whom the predominant mode of red-cell clearance was extravascular

(Ekvall *et al*, 2001). G6PD deficiency was uncommon and no unstable haemoglobin was detected.

Muscle-cell injury was common in this series, and plasma myoglobin and CK levels were greater in Groups I and II than Group III. Urine myoglobin was greater in Group I than Groups II and III. Levels of plasma myoglobin ranging from 300 ng/ml to 15000 ng/ml have been reported to be consistent with dark urine (Singh *et al*, 2005; Davis *et al*, 1999). Certainly, plasma myoglobin levels above 300 ng/ml were observed in many children in Group I. Plasma myoglobin was greater in children with cerebral malaria, hyperlactataemia and those who died. It was associated with plasma creatinine but not with acidosis. Plasma CK and CKMB levels were greater in children who died than those who survived.

There was evidence of chronic haemolysis in only 2/22 Group I children at follow-up. One child had probable tropical splenomegaly syndrome and no underlying cause of haemolysis could be identified in the other.

The passage of dark urine in children with malaria was not uncommon in this series but occurred infrequently in children admitted to hospital with a primary diagnosis other than malaria. Although it was not possible to measure urine haemoglobin levels under field conditions, it is likely that both haemoglobin and myoglobin contributed to dark urine.

To my knowledge, there were no reports in the literature prior to this study of dark urine in children with malaria in Papua New Guinea. Quinine-induced haemolysis was a major cause of BWF in Vietnamese adults (Tran *et al*, 1996),

Senegalese children (Rogier *et al,* 2003) and European expatriates (Van den Ende *et al,* 1998; Bruneel *et al,* 2001). However, haemolysis following re-exposure to quinine could have occurred in only 3 children in the PNG series, who had previous exposure to malaria and were DCT positive. Similarly, G6PD deficiency was also a major cause of BWF in Vietnamese adults (Tran *et al*, 1996). However, in the PNG series, G6PD deficiency was not found in any child who had passed dark urine.

Prior to my study, levels of urine myoglobin had not been reported in children with malaria and although dark urine was attributed to myoglobinuria in adults with malaria from Sri Lanka (De Silva *et al,* 1988), Indonesia (Sinniah & Lye, 2001) and Nigeria (Knochel & Moore, 1993), urine myoglobin concentration was not determined in any of these studies. Although levels of urine myoglobin were not as high (300,000 ng/ml) as those proposed by Beetham (2001) as being consistent with dark urine, they were nevertheless greater in children in Group I and were consistent with those reported in Sri Lankan adults with dark urine and rhabdomyolysis following snake-bite (Phillips, 1988). Also, myoglobinuria may have been the major cause of dark urine in those children with normal plasma haemoglobin levels.

In children admitted with malaria in this study, muscle-cell injury was more common (61%) than in studies in children from The Gambia (45%) and Ghana (45%) (Miller *et al,* 1989; Erhardt *et al*, 2005). Similarly, elevated levels of CK were found in 73% of children in this study compared to 28% in The Gambia (Miller *et al*, 1989). Cardiac muscle injury (CKMB > 2.5% total CKMB) was also

more common in this series than in The Gambia but is consistent with the Ghanaian study where elevated CKMB levels occurred in children with severe malaria and those who died (Ehrardt *et al*, 2005). The greater degree of muscle-cell injury observed in my study compared to other studies, may explain the greater frequency of dark urine in this series.

The association between muscle-cell injury and cerebral malaria suggests sequestration of parasitized red cells occurs simultaneously in brain and muscle. Muscle biopsies were not collected in this study but sequestration of *P. falciparum* was observed in muscle biopsies of 36 Thai adults (Davis *et al*, 1999). It is also possible that hyperlactataemia, the major predictor of fatal outcome in the PNG series, may have resulted from the breakdown of skeletal muscle protein as well as tissue hypoxia.

My findings highlighted the importance of muscle-cell injury in children with malaria and provided greater insight into the cause of "blackwater fever" in children.

4.3 α^+- thalassaemia

Previous research had described a positive association between the frequency of α^+-thalassaemia and transmission of falciparum malaria in this region. This suggested that α^+-thalassaemia persists in this population because it affords a survival advantage against malaria. The interaction between α^+- thalassaemia and malaria was studied in children and in pregnant women. The studies were reported in PNAS IN 1997 and the British Journal of Haematology in 2006 and are described below.

3. Allen SJ, **O'Donnell A**, Alexander NDE, Alpers MP, Peto TEA, Clegg JB, Weatherall DJ. α^+-thalassaemia protects children against disease due to malaria and other infections. *Proceedings of the National Academy of Sciences USA*, 1997; 94: 14736-14741.

In this prospective case-control study, the protective effect of α^+-thalassaemia against severe malaria in children as a surrogate for malaria mortality was assessed. This was the first investigation of the interaction between α^+-thalassaemia and malaria in this population. Children were eligible to enroll into the study if they had lived in Madang province for at least one year. Index cases were children admitted to Madang Hospital with one or more manifestation of severe malaria (severe anaemia, coma, hypoglycemia, acidosis and hyperlactatemia) as defined by WHO criteria (described above in paper 1). An age, ethnicity, village and sex-matched community control was recruited for each

index case. Children admitted to the pediatric ward with a primary diagnosis other than malaria were recruited as severe "non malaria" controls. Children were also recruited from local health clinics: those with fever and parasitaemia were classified as "mild malaria" and those with a febrile illness without parasitaemia were classified as "mild non-malaria". A venous blood sample was collected from all children participating in the study. For the purpose of determining the gene frequency of α^+-thalassaemia in the normal population, blood samples were collected from healthy adult volunteers and blood donors from Madang. In order to assess the frequency of non-deletional α^+-thalassaemia cord blood samples were collected from infants delivered in Madang hospital.

Figure 11: Typical PNG village and field worker recruiting a community control child.

The methods used for blood collection and the laboratory investigations to assess the severe manifestations of malaria have been described above in paper 1. Further laboratory analyses included screening blood samples for G6PD deficiency and abnormal haemoglobin variants, measurement of HbA_2 (to identify carriers of β-thalassaemia) in all children and adult blood samples, and measurement of Hb Bart's in cord blood samples. DNA was extracted from white blood cell pellets and α-globin genotype and the band 3 deletion for SAO were determined by Southern blotting (Old & Higgs, 1983) and PCR (Jarolim *et al*, 1991), respectively.

Conditional logistic regression analysis with case/control status as the outcome variable was performed for all index case –community control pairs in whom α-globin genotype had been determined for both members of the pair. The frequency of α-globin genotype in the other clinical groups was compared with that in the community control group. Age, ethnicity and residence were included as possible confounders. All analyses were repeated to include the band 3 deletion for SAO as a possible confounder. All regression analysis was done using EGRET. The Kruskall-Wallis test was used to compare continuous laboratory variables with α-globin genotype and the χ^2 test used to compare α-globin genotype with clinical categorical variables in all children with malaria. P values < 0.05 were considered significant.

The frequency of $-\alpha$ was 0.73 in 215 adult volunteers from Madang and was 0.68 in 225 cord blood samples from infants of whom both parents were of Madang ethnicity. The frequency of $-\alpha$ varied markedly according to ethnicity and was 0.0 in 13 infants of whom both parents were of highland ethnicity, 0.35 in 26 infants of whom both parents were from other coastal regions and was 0.87 in infants of whom both parents were of Sepik ethnicity. The frequency of non-deletional α^+-thalassaemia was 1.1%. The frequency of $-\alpha$ was similar in children admitted to hospital with and without malaria (0.80 and 0.74 respectively), in those attending health centres with mild malaria and mild non-malaria disease (0.83 and 0.80 respectively) and 0.87 in the community controls. The band 3 deletion for SAO was present in 6.9% of children and was not associated with α^+-thalassaemia. Studies of the interaction between the SAO band 3 deletion and malaria are reported separately (see below). Other red-cell variants associated with protection against malaria were rare; 4 children (0.3%) had raised HbA_2 levels consistent with β-thalassaemia trait, 8 children (0.6%) had HbJ Tongariki, and G6PD deficiency occurred in 13 (2.3%) boys.

α-globin genotype was determined in both members of 249 index case –control pairs. Compared with the community controls, the risk of severe malaria was 0.40 (95% confidence interval (CI), 0.22-0.74) in α^+-thalassaemia homozygotes and 0.66 (95% CI, 0.37-1.20) in heterozygotes. When analysed according to severe malaria manifestation, α^+-thalassaemia homozygotes were protected against all manifestations of severe malaria except for coma and hypoglycaemia. The lowest odds ratios for α^+-thalassaemia homozygotes were recorded in the

acidosis and hyperlactatemia subgroups -the two complications shown previously to be most predictive of malaria mortality (see paper 1 above). In those children admitted to hospital with malaria, haemoglobin and biochemical indices did not differ significantly according to α- globin genotype. Although the mechanism of protection against malaria is unknown and several mechanisms have been proposed (see introductory chapter), the fact that *P. falciparum* parasite frequency and density were similar in cases and controls, suggests that α^+-thalassaemia does not impair the blood stage of the infection.

Surprisingly, the risk of hospital admission in 233 children with infections other than malaria was also reduced to a similar degree in α^+-thalassaemia homozygotes (0.36; 95% CI, 0.22-0.60) and heterozygotes (0.63; 95% CI, 0.38-1.07). It is possible that α^+-thalassaemia may have reduced disease caused by non-malaria infections as a result of the prevention of acute malaria and the resulting immunosupression, or possibly as a consequence of protection against severe malaria anaemia. It is also possible that an enhanced anti-malaria immunity in α^+-thalassaemia contains a non-specific immunological component which also reduces other disease.

The remarkably high frequency of α^+-thalassaemia and the protective effect it affords against malaria and some other infections, suggests that it is likely to have a major impact on child survival in coastal Papua New Guinea.

Prior to this research, the prevalence of α-thalassaemia in the Madang region had been estimated in three studies. Oppenheimer *et al* (1984) detected raised levels of Hb Bart's in 175/217 (81%) cord blood samples collected from Madang

hospital. Using DNA analysis, Oppenheimer *et al* (1984) reported that 27/30 (90%) infants and adults from Madang had either the -*α*/*αα* or -*α*/-*α* genotypes. Yenchitsomanus *et al* (1986) analysed 75 samples obtained from adults from 6 villages around Madang and from 19 blood donors and reported a genotype frequency for -*α* of 0.79. Flint *et al* (1986) analysed 63 samples from subjects attending schools and hospitals in the north coastal region of PNG (Madang, East and West Sepik) and reported a genotype frequency for -*α* of 0.68. I determined the frequency of -*α* in over 1700 DNA samples collected in this study. The frequency of -*α* varied markedly according to ethnicity, but the frequency of –*α* in those of Madang ethnicity was very similar to that reported previously. Similarly, the absence of α^{+}-thalassaemia in 13 infants of highland ethnicity supports the findings of Flint *et al* (1993) that α^{+}-thalassaemia is very uncommon in the non-malarious highlands of PNG.

Although studies to assess the protective effect of Hb variants such as sickle-cell trait against malaria had been undertaken, this was the first case-control study to investigate the interaction between *α*-thalassaemia and severe malaria and was the first to demonstrate a protective effect of α^{+}-thalassaemia against malaria.

4. **O'Donnell A**, Raiko A, Clegg JB, Weatherall DJ, Allen SJ. α^+-thalassaemia and pregnancy in a malaria endemic region of Papua New Guinea. *British Journal of Haematology* 2006; 135: 235-41.

Malaria is an important cause of maternal anaemia and low birthweight, particularly in primigravidae (Brabin, 1991). In view of the high frequency of α^+-thalassaemia in the Madang population and its strong protective effect against malaria in children (described above), I investigated the possible protective effect of α^+-thalassaemia against malaria during pregnancy. However, α^+-thalassaemia may also have deleterious effects on pregnancy outcome. It results in a deficiency of α-globin chains. In the developing fetus, α^+-thalassaemia may impair oxygenation which could result in early miscarriage. Therefore, I also studied the effect of maternal α^+-thalassaemia on reproductive fitness and pregnancy outcome.

Women with live singleton infants were eligible for the study if they had lived in Madang Province throughout their pregnancy. All primigravidae and an equal number of second gravidae and multigravidae were recruited within 24 hours of delivery. Information regarding reproductive fitness (age in primigravidae, gravidity, pregnancy interval and the number of miscarriages and stillbirths) was recorded and verified with ante-natal cards. Details of haemoglobin measurements taken during ante-natal visits were also recorded. Following delivery, infants were weighed and examined. Gestational age was determined

by a rapid score and infants with low birthweight were assessed for prematurity. Venous blood was collected into EDTA from each mother and a sample of cord blood collected for each infant. Laboratory investigations included full blood count, examination of Giemsa stained maternal peripheral blood and placental films for malaria parasites and measurement of plasma ferritin. DNA was extracted from maternal and cord blood cell pellets and α-globin genotype was determined.

α-globin genotype was determined in 913/987 (92.5 %) women and 489/498 (98.1%) infants recruited into the study. The frequency of - α was 0.61 in all women and 0.65 in those of Madang ethnicity. The frequency of – α was 0.61 in the infants.

Maternal age in primigravidae, gravidity, and interval between pregnancies did not vary significantly according to α-globin genotype, indicating that maternal α^+-thalassaemia did not impair fertility in this series of women. Similarly, there was no association between previous fetal loss (miscarriage or stillbirth) and α-globin genotype. Pregnancy outcome, as measured by the incidence of preterm delivery and low birthweight was similar in mothers with and without α^+-thalassaemia. As expected, during pregnancy and following delivery, median haemoglobin concentration was significantly lower in women with α^+-thalassaemia compared to normals. Similarly, median plasma ferritin concentration was also significantly lower in women with α^+-thalassaemia compared to normals. However, these findings did not result in an adverse outcome of pregnancy.

Malaria was common and approximately 20% of placental blood films contained *P. falciparum* parasites. In primigravidae, the group of women most susceptible to malaria, there was no association between α-globin genotype and the frequency of placental malaria infection or malaria infection and *P. falciparum* density in the peripheral blood after delivery. Similarly, in multigravidae, placental malaria and *P. falciparum* density in peripheral blood after delivery did not vary according to α-globin genotype. Thus, there was no evidence of a protective effect of α^+-thalassaemia against malaria, nor did α^+-thalassaemia appear to affect parasite sequestration in the placenta. As expected, anaemia was common and women with malaria were more anaemic than those without.

There was no association between infant α-globin genotype and frequency of preterm delivery, birthweight and cord blood parasitaemia. As in the mothers, haematological indices in cord blood were associated with infant α-globin genotype: haemoglobin concentration, mean cell volume and mean cell haemoglobin were lower and erythrocyte count higher in infants with α^+-thalassaemia compared with normals.

Prior to this research, studies which assessed the interaction between α^+ thalassaemia and malaria during pregnancy were few. In a study of α^+ thalassaemia in 2326 pregnant women of diverse ethnic origins in the United Arab Emirates (UAE), a non-malarious area, White *et al* (1985) reported no deleterious effects on placental function, fetal development or maternal and fetal morbidity. Since anaemia and iron deficiency have been reported to increase the

risk of low birthweight in primigravidae (Brabin *et al,* 1990 a), it is possible that α^+ thalassaemic mothers would be at higher risk of low birthweight. However, in my study, malaria associated anaemia was not associated with α-globin genotype and I did not find an association between maternal α^+ thalassaemia genotype and birthweight. Interestingly, in a cross-sectional survey of Ghanaian women attending ante-natal clinics, malaria anaemia was marginally less in α^+ thalassaemic women than in normals (Mockenhaupt *et al*, 2000).

My finding of lower plasma ferritin levels in women with α^+ thalassaemia was surprising and was in contrast to studies in the UAE (White *et al,* 1986) in which plasma ferritin was similar in 50 women with normal α-globin genotype and 42 with α^+ thalassaemia. These findings are difficult to compare, since the UAE is a non-malarious area and α^+ thalassaemia was diagnosed using haematological findings rather than genotyping. Although the increased red-cell turnover in α^+ thalassaemia would be expected to lead to higher iron stores, in studies in children with malaria, I also found plasma ferritin levels were lower in children with α^+ thalassaemia than in normals (reported above). Since ferritin is also an acute phase protein, it is possible that the lower ferritin levels reflect a reduced acute phase response in α^+ thalassaemia.

In summary, this study was the first to investigate the interaction between α-thalassaemia and malaria in pregnancy and pregnancy outcome in PNG. α^+ thalassaemia did not protect against malaria during pregnancy nor did it impair

fertility. Similarly, the mild anaemia associated with α^+ thalassaemia does not appear to have a significant adverse effect on preterm delivery or birthweight.

4.4 Southeast Asian Ovalocytosis

The high frequency of SAO, determined by examination of thin blood films alone, reported in the malarious lowland and coastal regions of PNG (Serjeantson *et al* 1977; Cattani *et al, 1987),* prompted me to investigate the interaction between SAO and malaria in children and pregnant women. With the advent of molecular biology, it was possible to accurately determine the frequency of SAO in the Madang population and to describe the haematological indices and features of red-cell morphology best associated with SAO. These studies were reported in the British Journal of Haematology in 1999, and the American Journal of Tropical Medicine and Hygiene in 1999 and 2007 and are described below.

5. **O'Donnell A**, Allen SJ, Mgone CS, Martinson JJ, Clegg JB, Weatherall DJ. Red-cell morphology and malaria anaemia in children with Southeast Asian ovalocytosis band 3 in Papua New Guinea. *British Journal of Haematology* 1998; 101: 407-412.

This paper describes the haematological indices and red blood cell morphology associated with the band 3 deletion for SAO in children with and without malaria. Children recruited as part of the case-control study to investigate the interaction between α^+ thalassaemia and malaria were investigated. The methods used for study enrollment, blood collection and laboratory investigations including full blood count, examination of Giemsa stained thick blood films for malaria parasites, haemoglobin electrophoresis, measurement of plasma bilirubin and

genotyping of DNA samples for α^+ thalassaemia and the band 3 deletion for SAO are described above in papers 1 and 3. Additionally, Giemsa stained thin blood films were prepared and examined by light microscopy. The number of ovalocytes per 1000 erythrocytes was counted and any abnormal features of red-cell morphology were recorded. These included the presence of:

i. Stomatocytes - red cells with a central slit-like region of pallor.

ii. Knizocytes - red cells with two slit-like regions of pallor separated by a narrow well-haemoglobinised region.

iii. Red cells with multiple irregular regions of pallor.

iv. Schistocytes - fragmented red cells of varying shapes, often with thorn-like projections around the cell.

v. Elliptocytes - cigar shaped red cells with a length to width ratio >2:1

vi. Target cells - red cells with a darker stained central area of haemoglobin separated from an outer ring of haemoglobin.

vii. Spherocytes - deeply stained red cells with a spherical (rather than disc-like) shape and lacking a central region of pallor.

viii. Reticulocytosis - Increased numbers (>2.5%) of reticulocytes (young red cells) which are slightly larger than normal and have a bluish hue.

Statistical analyses included the χ^2 test for categorical variables and multiple linear regression analysis which included - α globin genotype in the model (to account for the effect of α^+ thalassaemia on haematological indices), to assess the effect of SAO on haematological indices.

SAO genotype was determined in 848 children and the frequency varied markedly with ethnicity. SAO was present in 16/241(6.6%) community controls and 32/389 (8.2%) children admitted to hospital with *P. falciparum* malaria. It occurred in 7.1% of children of Madang ethnicity, 1% Sepiks and 0% in children of highland and other coastal region ethnicity. Examination of thin blood films showed that the red-cell morphological feature most consistent with SAO was the presence of multiple linear or irregular pale regions within red cells. This abnormality was observed in the thin films of 93.8% of community control children with SAO and 87.5% of children with SAO who had acute malaria. Other red-cell morphological features associated with SAO included the presence of stomatocytes and elliptocytes. Surprisingly, the proportion of ovalocytes present in thin films was a less sensitive indicator of SAO. No ovalocytes were found in the thin films of 6 children with SAO (4 community controls and 2 with acute malaria) and the thin films of 13 children with SAO (1 community control and 12 with acute malaria) had less than 20% ovalocytes present.

Since α^+ thalassaemia is very common in this population, haematological indices consistent with microcytic anaemia (ie. low haemoglobin concentration and low mean cell volume) are found in many children, thus making it very difficult to directly assess the effect of SAO on these indices in this population. However, multiple regression analysis which included - α globin genotype in the model showed that SAO did not alter haematological indices in community controls.

However, in children with malaria, haemoglobin concentration and red-cell count were significantly lower and mean cell volume and platelet count were significantly higher and reticulocytes more common, in children with SAO compared to normals. The degree of ovalocytosis was significantly less in children with SAO and malaria, compared to community controls with SAO but without malaria.

The frequency and density of *P. falciparum* infection in community controls was similar in children with and without SAO. Also, there was no evidence that SAO protected against hospital admission or clinic attendance in children with malaria, since the frequency of SAO in children with malaria and in community controls was similar. However, a negative association between SAO and cerebral malaria was found when the interaction between SAO and the severe manifestations of malaria in children was investigated. This study is described below.

Previously, SAO was diagnosed by characteristic alterations in red-cell morphology (Amato & Booth, 1977) and by the degree of ovalocytosis present in thin blood films, with cut-off levels ranging from 25 to 50% (Mgone *et al*, 1996). My study led to the first publication to describe the red-cell morphology most consistent with SAO genotype. I found that there were several children with SAO whose blood films did not contain any ovalocytes or who had <20% ovalocytes present. Using the previous criteria, SAO would have been missed in these children. SAO homozygosity is lethal and, therefore, improved criteria for the

diagnosis of SAO heterozygotes based on examination of thin blood films will help in genetic counselling in those parts of the world where molecular diagnosis is not available.

Previous studies in PNG to assess the interaction between SAO and malaria gave conflicting results. Serjeantson *et al* (1977) found no consistent differences in parasite rates in children with and without SAO, whereas Cattani *et al* (1987) found that the frequency of malaria parasitaemia was less in children who had >50% ovalocytes present in thin films than children with <30% ovalocytes. Using molecular techniques to diagnose SAO, my findings of similar frequencies and density of *P. falciparum* infection in community control children with and without SAO, are in agreement with Serjeantson *et al* (1977).

My study was the first to describe the greater degree of anaemia in SAO children with malaria than in normals, and since the degree of ovalocytosis was lower in children with SAO band 3 during acute malaria, it is possible that a selective loss of ovalocytes may contribute to malaria anaemia in SAO.

6. Allen SJ, **O'Donnell A**, Alexander NDE, Mgone CS, Peto TEA, Clegg JB, Alpers MP, Weatherall DJ. Prevention of cerebral malaria by Southeast Asian ovalocytosis in children in Papua New Guinea. *American Journal of Tropical Medicine and Hygiene* 1999; 60: 1056-60.

This publication is an extension of the previous studies (3 and 5) and describes the relationship between SAO and the common severe manifestations of malaria in children, as a surrogate for malaria mortality. Details of the study site, children recruited and the clinical and laboratory investigations performed in this prospective-case control study have been described in papers 1, 3 and 5 above.

The frequency of SAO was determined in children admitted to hospital and attending local health clinics with and without malaria and in community controls who were individually matched to each severe malaria case for age, sex, ethnicity, village and season. The frequency of SAO in severe malaria cases was compared with that in community controls. In children admitted to hospital with malaria, the association between SAO and severe manifestation of disease (severe anaemia, cerebral malaria, acidosis and hyperlactataemia) was investigated.

SAO band 3 status was determined in 1,224 children. The frequency of SAO was 5.9% in 307 community controls, and in children admitted to hospital was 6.3% in 431 children with malaria and 5.8% in 240 children without malaria. The frequency of SAO in children attending local health clinics was 6.4% in 110

children with malaria and 5.1% in 136 children without malaria. The risk of clinical malaria was not associated with SAO genotype, as assessed by logistic regression analysis (which included - α globin genotype and demographic variables in the model). These findings suggest that, unlike α^+ thalassaemia, SAO does not reduce mortality due to non-malaria infections. Similarly, SAO did not prevent mild disease, since the frequency of SAO was similar in children attending local health clinics and in community controls.

When severe manifestations of disease were compared to SAO status in children admitted to hospital with malaria, a remarkable finding was the complete absence of SAO in 68 children with cerebral malaria and for whom complete data were available compared with 6/68 (8.8%) individually matched controls. Median haemoglobin concentration in children with malaria was 4.8g/dl and 6.0g/dl in children with and without SAO, respectively (see paper 5 above). However, analysis of 173 case-control pairs showed that the increased frequency of SAO in children with malarial anaemia compared to community controls was of borderline statistical significance. SAO was not associated with other manifestations of severe disease including acidosis or hyperlactataemia.

Previously, SAO had been shown to protect against cerebral malaria in children from the same region of PNG. SAO was absent in 35 children with cerebral malaria but was present in 15/103 unmatched population controls (Genton *et al,* 1995). When both studies are considered together, SAO appears to provide remarkable protection against cerebral malaria. The pathogenesis of cerebral malaria is thought to be due in part, to the sequestration of erythrocytes

parasitized by *P. falciparum* in the brain microvasculature, and the band 3 protein has been implicated in this process. *In vitro* studies have shown that infection of erythrocytes with *P. falciparum* causes modifications in the band 3 protein on the red-cell surface (Crandall *et al,* 1994) and synthetic peptides based on the amino acid sequences that cause these modifications have been shown to be associated with reduced adherence of *P. falciparum* infected erythrocytes to melanoma cells *in vitro* and prevented sequestration when infused into Aotus and Samiri monkeys with *P. falciparum* infection (Schofield *et al,* 1992). It is possible that the 27 base pair deletion of band 3 SAO may also result in similar modifications and reduce the expression of cytoadherence ligands on the red-cell surface.

In summary, SAO was shown to confer potent protection against cerebral malaria in children in PNG. However, this selective advantage was balanced by non-viability in the homozygous state and an exacerbation of malaria anaemia in the heterozygous state. Further studies to explore the possible mechanism of protection against cerebral malaria were advocated.

7. **O'Donnell A**, Raiko A, Clegg JB, Weatherall DJ, Allen SJ. Southeast Asian ovalocytosis and pregnancy in a malaria endemic region of Papua New Guinea. *American Journal of Tropical Medicine and Hygiene* 2007; 76: 631-33.

The findings that SAO provided potent protection against cerebral malaria in children prompted me to investigate the possible protective effect of SAO against malaria in pregnancy. This short report is described below.

During pregnancy, particularly in first pregnancy, women are more susceptible to malaria, and this can result in miscarriage, maternal anaemia and low birthweight. Much of the pathology associated with malaria in pregnancy is due to sequestration of parasitized erythrocytes in the intervillous space of the placenta (Whitty *et al,* 2005). Since sequestration of parasitized erythrocytes in the brain and the placenta are the main cause of pathogenesis in cerebral malaria and malaria in pregnancy respectively, and in view of the potent protection that SAO provided against cerebral malaria in children, I investigated the effect of SAO on reproductive fitness, malaria in pregnancy and pregnancy outcome.

Women with live singleton infants were eligible for the study if they had lived in Madang Province throughout their pregnancy. Details of the women recruited into this study, information collected regarding reproductive fitness, haemoglobin measurements taken during ante-natal visits, clinical assessment, blood sample collection, preparation of placental thick and thin films and the laboratory

investigations performed in this study have been described above in paper 4. Additionally, maternal DNA samples were genotyped for the SAO band 3 deletion by PCR (Mgone *et al,* 1996).

927 maternal DNA samples were genotyped for the SAO band 3 deletion. SAO was present in 81/927 (8.7%) women. Age in primigravidae, pregnancy interval in multigravidae and previous fetal loss were similar in women with and without SAO. As expected, peripheral and placental malaria infection occurred more frequently in primigravidae than multigravidae. However, there was no association between SAO and the frequency and density of *P. falciparum* infection in the peripheral blood or in the placenta following delivery. Median haemoglobin concentration was similar in women with and without SAO both during pregnancy (10.2 g/dl and 9.6 g/dl, respectively) and after delivery (10.5g/dl and 10.7 g/dl respectively). Severe anaemia (Hb< 7g/dl) was uncommon in this series and was not associated with SAO. Median birthweight in term infants and the frequency of preterm delivery, and low birthweight were not associated with maternal SAO genotype.

I found no evidence that SAO impaired reproductive fitness in women in this study. Despite the striking finding of potent protection against cerebral malaria in children, I did not find that SAO protected against placental malaria in pregnancy. Since sequestration of parasitized erythrocytes is a feature of both cerebral and placental malaria, a possible explanation for this finding is the different host receptors for parasite antigens in the brain and placenta. Therefore, SAO may impair binding in the brain, but not in the placenta. This is in keeping with the

findings of Cortes *et al* (2005) who showed *in vitro* that adhesion to chondroitin sulphate A (the predominant host receptor in the placenta), is not impaired in people with SAO (Cortes *et al,* 2005). The greater degree of malarial anaemia observed in children with SAO was not found in pregnant women with SAO and malaria. Finally, SAO did not appear to affect the outcome of pregnancy.

Prior to publication of my findings, a community based control study of placental malaria in women with SAO from Madang was published (Benet *et al*, 2006). My findings that SAO was not associated with either anaemia in pregnancy, birthweight or frequency of low birthweight were in agreement with those of Benet *et al* (2006). However, in contrast to my findings, Benet *et al* (2006) found that placental malaria was less common in women with SAO than in controls, and the reason for this discrepancy is unclear. Since SAO was not associated with birthweight in either study, this would suggest that the reduction of placental parasitaemia observed by Benet *et al* (2006) would be of little clinical benefit. A further difference between the two studies was that Benet *et al* (2006) observed a lower frequency of SAO in pregnant women than in non- pregnant women, and proposed that SAO, in conjunction with another genetic trait causes sterility. However, I think that this is unlikely, since the frequency of SAO in pregnant women attending hospital for delivery was similar to that in healthy young girls living in the same community. My finding that placental malaria was not associated SAO genotype suggests that adherence of parasitized erythrocytes to placental host receptors such as chondroitin sulphate A is not impaired by SAO. In summary, although an interaction between SAO and malaria during pregnancy

can not be excluded, I found no evidence that it provided a clinical benefit in this hospital-based cohort of women.

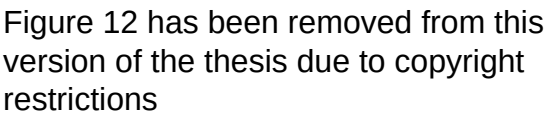


Figure 12: Traditional perceptions of childbirth; a local artist's impression.

Chapter 5 Significance and relevance of this programme of research to subsequent studies

The series of research studies described above represent a comprehensive investigation of malaria in children and pregnant women and its interaction with α^+ thalassaemia and Southeast Asian ovalocytosis in PNG. Using molecular techniques and large numbers of subjects, reliable estimates of the frequencies of α^+ thalassaemia and SAO were determined in over 2000 adults, children and infants from the Madang study area (papers 3, 4, 5,6 & 7). The clinical and laboratory manifestations associated with severe malaria in children were described (paper 1) and muscle-cell injury was identified as a cause of dark urine (paper 2). α^+ thalassaemia and SAO were shown to protect against severe malaria and cerebral malaria, respectively, in children (papers 3 & 6). Additionally, α^+ thalassaemia also protected against hospital admission in children with non-malarial illnesses (paper 3). However, in studies in pregnant women, neither α^+ thalassaemia nor SAO were shown to protect against placental or peripheral malaria at delivery, or to improve pregnancy outcome (papers 4 & 7). Finally, by identifying SAO using molecular techniques it was possible to improve the laboratory description of the red-cell morphology associated with SAO (paper 5). In this section I will discuss the importance of these findings to the subsequent research in this field and the implications of this research for future studies.

The description of severe malaria in children in PNG was amongst the first to identify metabolic acidosis, particularly due to hyperlactatemia, as an important feature of severe malaria and a major predictor of death (paper 1). This study also supported the idea that the manifestations of severe malaria did not fall neatly into the classification of severe malaria anaemia or cerebral malaria but consisted of other, often overlapping, features. Further studies in Kenyan children and Thai adults have highlighted the importance of acidosis and its associated pathophysiology in severe malaria (English *et al*, 1997; Day *et al*, 2000) and have proposed that acidosis occurs as a result of several factors including tissue hypoxia, impaired liver function and impaired processing of bicarbonate by the kidneys (Day *et al*, 2000). Additionally, Maitland *et al* (2003) have suggested that hypovolaemia is another feature of severe malaria in children which may contribute to acidosis. These studies have enabled researchers in Kenya to describe more accurately the diverse pathophysiology of severe malaria in children (Maitland & Marsh, 2004).

The unexpected finding of the frequent occurrence of dark urine in children with malaria in PNG, and its association with muscle-cell injury (paper 2) raises several important questions. It may be that dark urine in children with malaria also occurs in other malarious areas but has been overlooked previously. Indeed, in studies currently on-going in children with malaria in Nigeria, dark urine has also been identified (Professor Olugbemiro Sodeinde - personal communication). Due to field conditions, I was not able to quantify the amount of haemoglobin in urine samples and therefore could not definitively determine the relative

contribution of haemoglobin and myoglobin to dark urine. However, I am currently collaborating with an on-going study in children with malaria in PNG and hope to clarify this by using mass spectrometry to quantify both haemoglobin and myoglobin in urine samples.

The striking protection afforded by α^+ thalassaemia against severe malaria in children in PNG (paper 3) prompted others working in East and West Africa to investigate this association. In Ghana, in an unmatched case-control study of 301 children with severe malaria and 2107 controls, Mockenhaupt *et al* (2004) found that heterozygotes for α^+ thalassaemia were protected against severe malaria: 32.6% of control children compared to only 26.2% of severe malaria cases were heterozygous for α^+ thalassaemia (odds ratio [OR], 0.74; 95% confidence interval [CI], 0.56-0.98). However, Mockenhaupt *et al* (2004) did not observe a protective effect against malaria in children with homozygous α^+ thalassaemia.
In a study of 655 Kenyan children with severe malaria and 648 controls matched for village and ethnicity, Williams *et al* (2005a) reported that the incidence of both heterozygous and homozygous α^+ thalassaemia was reduced in the cases compared with controls (adjusted OR 0.73 [95% CI 0.57-0.94] and 0.57 [0.40-0.81] respectively) and amongst severe malaria deaths compared with survivors (0.60 [0.37-1.00] and 0.37 [0.16-0.87] respectively). This study confirmed my earlier findings of a survival advantage against malaria in α^+ thalassaemia heterozygotes and homozygotes. In Kenya, α^+ thalassaemia protected against coma, severe anaemia and acidosis. In the PNG series, α^+ thalassaemia

protected against severe malaria anaemia and acidosis, but no association with coma was found (paper 3). It is possible that differences in the occurrence of other haemoglobinopathies (e.g. SAO in PNG and HbAS in Africa), explains this discrepancy.

The finding that α^+ thalassaemia also protected against hospital admission in children with non-malarial illnesses in PNG (paper 3) was surprising, but has subsequently received some support. Wambua *et al* (2006) studied the effect of α^+ thalassaemia on the incidence of other illnesses in a birth cohort of 2,104 Kenyan children and a mild disease cohort of 301 children. They found that children from the birth cohort with heterozygous α^+ thalassaemia were less likely to be admitted to hospital with severe anaemia than children with a normal α - globin genotype. In the mild disease cohort, the incidence of lower respiratory tract infection was less in children with heterozygous or homozygous α^+ thalassaemia compared to normals.

The striking findings regarding α^+ thalassaemia and malaria in the PNG case-control study has prompted further studies to investigate possible mechanisms of protection against malaria. Complement receptor 1 (CR1) is expressed on the surface of red cells and is associated with rosetting - a process by which uninfected red cells bind to *P. falciparum* infected red cells to form clumps *in vitro*. Rosetting is thought to contribute to the pathogenesis of severe malaria. In a collaborative study with Cockburn *et al* (2004) to determine the frequency of

polymorphisms in the CR1 gene (associated with CR1 deficiency and reduced rosette formation) in children in the case-control study, we found that CR1 expression is reduced in α^+ thalassaemia red cells and CR1 polymorphisms and α^+ thalassaemia independently protected against severe malaria. This study provided evidence that polymorphisms in the CR1 gene were selected in this population because of the protection they afforded against malaria.

Other studies to investigate the possible mechanism of protection against malaria in α^+ thalassaemia have investigated possible differences in the acute phase response in malaria in children according to α-globin genotype. In a cross-sectional survey of children in Madang, PNG, Imrie *et al* (2006), found that concentrations of the acute phase protein haptoglobin (Hp), were greater in children homozygous for α^+-thalassaemia compared to heterozygotes. These researchers hypothesized that greater Hp levels may reduce inflammation in malarial disease. I have collaborated with this research group and I have measured levels of the acute phase proteins (albumin, α1-anti-trypsin, C-reactive protein, ferritin and haptoglobin) in plasma samples collected from children in the case-control study cohort (manuscript in preparation).

In a further collaborative study, a simple mathematical model has been developed to explain the unexpected observation of the lower degree of malaria anaemia in children with α^+-thalassaemia compared to normals. It is suggested that following a similar degree of red-cell lysis due to malaria, children with α^+-

thalassaemia will lose less haemoglobin than children with a normal α- globin genotype because they have a greater number of red cells which contain less haemoglobin per cell compared to normals (manuscript submitted for publication to Public Library of Science).

Clearly, although several studies have identified potential mechanisms for the protection afforded against malaria by α^+- thalassaemia, these findings need to be confirmed in further studies and more research is required in this field.

The striking protection against cerebral malaria in children conferred by the band 3 deletion for SAO (paper 6) has prompted further investigations to study possible mechanisms. Cortes *et al* (2004), found that SAO erythrocytes were more resistant to invasion *in vitro* by 4 of 5 strains of *P. falciparum* (3D7-A). In a further study, Cortes *et al* (2005) found that the strains of *P. falciparum* which invaded SAO erythrocytes least effectively were those that bound to ICAM-1. This is a major receptor expressed on the vascular endothelium of the brain, and this finding suggests that the protection conferred by SAO may be due to resistance to a sub-set of parasite strains that are responsible for cerebral malaria. SAO erythrocytes also showed greater adhesion *in vitro* than normal erythrocytes to CD36 (a major endothelial receptor for sequestration). Since CD36 is not expressed in the brain, Cortes *et al* (2005) proposed that the sequestration of parasitized erythrocytes in tissues other than the brain in individuals with SAO affords protection against cerebral malaria. Despite these

interesting findings, the mechanism of protection afforded by SAO against cerebral malaria requires further investigation. A priority is the assessment of the relevance of findings in *in vitro* studies to clinical disease in children.

The rationale for investigating the interactions between red-cell polymorphisms in malaria during pregnancy was to identify possible deleterious effects. This was because common red-cell polymorphisms are thought to have some deleterious effects as they have not progressed to fixation. Given the protective effect of common red-cell polymorphisms against severe malaria in children, an alternative possibility is that they may also have beneficial effects in pregnancy. My studies of a large number of pregnant women failed to identify either beneficial or deleterious effects of α^+- thalassaemia and SAO on malaria during pregnancy (papers 4 & 7). It is possible, that other, as yet unidentified, red-cell polymorphisms may modify the effect of α^+- thalassaemia and SAO during pregnancy. Indeed, in studies in Kenya, Williams *et al* (2005b) have shown that when the - α globin gene is co-inherited with HbS, the protective effect that each of these polymorphisms usually affords against severe malaria in children is lost. Therefore, further studies are warranted in this field.

One major initiative that will try to address some of the issues raised above is the Malaria Genomic Epidemiology Network (MalariaGEN) in which DNA samples, collected from research groups from more than 15 malaria –endemic countries will be screened for up to one million single nucleotide polymorphism markers (SNP). The results collected from many different populations will then be used to

investigate gene-environment interactions and will provide greater insight into the genomic epidemiology of malaria (Kwiatkowski, 2005). The DNA samples collected from the PNG case-control study will be included in this initiative.

Finally, since SAO homozygosity is lethal and molecular diagnosis is often not available in low resource settings, I hope that my detailed description of the red-cell morphology associated with SAO (paper 5) may facilitate improved diagnosis of SAO heterozygotes, and be of benefit in genetic counselling.

Over 1.2 million deaths, predominantly in children under the age of 5 years, occur each year as a consequence of malaria. This research has demonstrated potent protection against severe malaria in children by α^+ thalassaemia and SAO (papers 3 & 6). These findings have already prompted investigation of the mechanisms that underlie the protection afforded by these common red-cell polymorphisms. Further delineation of protective mechanisms may offer new approaches to prevention and treatment of this devastating disease.

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To Whom It May Concern:

20th August, 2007

I have read the commentary of the work of Mrs. Angela Allen (nee O'Donnell) written as evidence for her application for the degree of PhD. This is just to confirm that this is an excellent and completely accurate account of her published work and, in particular, that the accounts that she has given of her role in each of the studies that are described is absolutely accurate.

Yours faithfully,

D.J. Weatherall MD, FRCP, FRCPath, FRS
Regius Professor of Medicine Emeritus
University of Oxford
Chancellor, Keele University, UK

Swansea University
Prifysgol Abertawe

November 13th, 2007

To whom it may concern,

Re: Severe malaria in children in Papua New Guinea

Dear Sir / Madam,

This is to confirm that I have reviewed the thesis by Angela Allen (nee O'Donnell) and that the details given of her contribution to the research work undertaken in Papua New Guinea regarding the interaction between inherited red cell defects and malaria are accurate. As detailed in the thesis, Angela made highly significant contributions to all aspects of the work including the evaluation of severe malaria in children.

I would be delighted to provide any further information that may be required.

Sincerely,

Stephen Allen.

Stephen Allen, MD, MRCP (Paeds), FRCPCH, DTM&H; Reader in Paediatrics
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Appendix 3 has been removed from this copy of the thesis due to copyright restrictions. For a list of the published papers included for examination, please refer to the Table of Contents