An investigation of the protective effect of alpha+-thalassaemia against severe *Plasmodium falciparum* amongst children in Kumasi, Ghana

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Abstract

Background: The alpha^{+}-thalassaemias are the most common monogenic disorders of humans, characterised by microcytic and hypochromic anaemia. Their high frequency reflects selective advantage against death from *Plasmodium falciparum* malaria. The most common type of alpha+-thalassaemia amongst people of African descent is the -α$^{3.7}$ deletional type and affects 26-33% of Ghanaians. *Plasmodium falciparum* malaria is a major cause of mortality amongst children in sub-Saharan Africa. Unlike HbAS, HbAC and G6PD deficiency there remains debate about whether alpha+-thalassaemia protects against malaria and the mechanism for the protection. **Methods**: 1672 children of ≤10 years were recruited and individuals with G6PD deficiency, HbAC and Hb AS reported to protect against malaria were excluded. 732 children with *Plasmodium falciparum* were tested for Hb, RBC, MCV, MCH and parasite density. The subjects were then categorised into normocytic and microcytic using a cut off MCV value of 76fL and normochromic and hypochromic using a cut off MCH value of 25 pg. Microcytic hypochromic individuals were genotyped by Polymerase Chain Reaction for the -α$^{3.7}$ deletional thalassaemia mutation. **Results:** The frequency of *Plasmodium falciparum* malaria in the studied population was 54.1%. There was a frequency of 21.0% for the heterozygous (-α/αα) and 8.3% for the homozygous (-α/-α) alpha+-thalassaemia, resulting in a carriage rate (α/αα & -α/-α) of 29.3%. Among the microcytic patients, geometric mean parasite density (GMPD) values were lower in the presence of an alpha+-thalassaemia genotype (-α/αα GMPD 9015, n=126 and -α/-α GMPD 6852, n=49) compared to normal genotype (αα/αα GMPD 51794, n=358) ($p<0.001$).

Severe malaria (GMPD ≥100000/μL) was less prevalent in microcytic patients with an alpha+-thalassaemia genotype (-α/αα 11.9% and -α/-α 16.3%) than either normocytic patients or microcytic patients with a normal genotype (32.9% and 53.6% respectively) ($p<0.03$). GMPD values were lower in hypochromic alpha+-thalassaemia genotypes (-α/αα GMPD 1728, n=44 and -α/-α GMPD 7160, n=23) compared to normal genotype (αα/αα GMPD 48997, n=141) ($p<0.001$), and individuals with Hb > 5 g/dL had lower GMPD compared to the severely anaemic (Hb ≤ 5 g/dL) ($p<0.001$). The differences in severe *Plasmodium falciparum* parasitaemia as well as the GMPD between children ≤ 60 and > 60 months for both the homozygous and heterozygous alpha^{+}-thalassaemia were not significant at p=0.399 and p=0.207 respectively. **Conclusion**: The severity of *Plasmodium falciparum* parasitaemia measured, as either GMPD or prevalence of severe parasitaemia was significantly lower in both the -α/αα and -α/-α- groups compared to microcytic individuals with normal genotype. Even though GMPD differed significantly amongst all alpha+-thalassemia genotypes, it was not driven by hypochromasia. Among the homozygous and heterozygous alpha+-thalassaemias, children with severe anaemia had a significantly high GMPD than their counterparts who were not severely anaemic making them more susceptible to severe malaria anaemia. No loss of protection was seen in children younger or older than 60 months and therefore the protective effect from severe malaria might not wane with age. The mechanism of protection from severe *Plasmodium falciparum* malaria is not clear, however the influence of microcytosis and hypochromasia on parasite density requires more research.

1.0 Literature Review

1.1 INTRODUCTION

Severe *Plasmodium falciparum* malaria is a major cause of mortality in children below 5 years in sub-Saharan Africa. However, the susceptibility to the disease is varied and human red blood cell (RBC) polymorphism protection from *Plasmodium falciparum* malaria provides a classical example of natural selection. The fact that individuals carrying the genetic traits are partially protected against severe malaria underscores the high prevalence of the human RBC polymorphism in areas of high *Plasmodium falciparum* malaria (Weatherall and Clegg 2010) Examples of such mutations include inherited blood disorders such as sickle cell trait, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and the alpha and beta thalassaemias. The $-\alpha^{3.7}$ kb deletion alpha^{+}-thalassaemias, which are predominant in Africa, have been suggested to confer protection from severe *Plasmodium falciparum* malaria and both the homozygous and heterozygous alpha^{+}-thalassaemia has been implicated (Mockenhaupt *et al*., 2004a).

Of the hundreds of thousands of genetic mutations that have arisen in the global population however, the most harmful ones usually disappear by affecting an individual's ability to reproduce; the mutations are lost before carriers can pass them on to their children, whereas most mutations are maintained in the population in low frequencies (Modell and Darlison 2008).

Some mutations however, can give the carrier such a large survival advantage that the mutations become positively selected for, leading to their presence in high frequencies, in some populations, (Williams, 2006a).

These blood-related genetic disorders, it seems, are amongst some of the major challenges faced by health delivery systems, especially in developing countries (Hoffbrand *et al*., 2004); because of their genetic nature, most carriers of these disorders are not aware of their status except when tested. However, there are very few centres in most developing countries that are equipped to carry out such testing. Even where such facilities are available they are, more often than not, used for research purposes. This means that most of such carriers will be unaware of their status for the most part of their lives. The likelihood is that the diseased genes are inherited by offspring of parents who are carriers (Mentzer and Kan 2001). One of such blood-related genetic disorders is thalassemia, an inherited disorder of haemoglobin synthesis that results from an alteration in the rate of α and β globin chain production (Hendricks, 2003). The genes responsible for the production of the α-chains are located on chromosome 16 and associate with the β-globin genes located on chromosome 11, to form the normal adult haemoglobin (α_2/β_2), (Figures 1.1 and 1.3), which plays the pivotal role of transporting oxygen to the cells of the body to facilitate their cellular functions (Yaish, 2005).

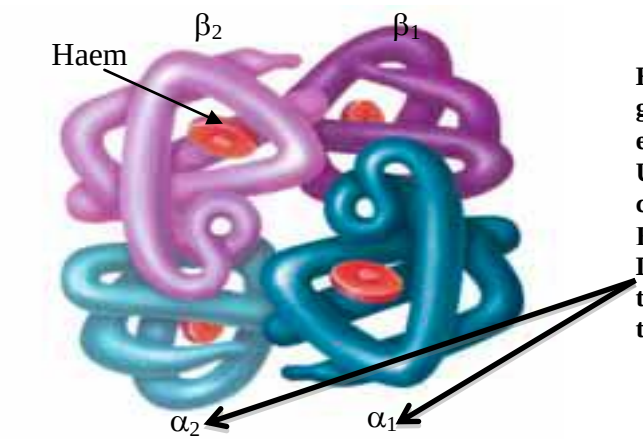

Figure 1.1: The haemoglobin molecule: note four haem molecules tucked inside globin chains. (Adapted from Ciela Betty, 2007)

Alpha thalassemia is a hereditary autosomal disorder resulting from the deficient expression of the functional α1 and/or α2 globin gene and is characterised by microcytic hypochromic anaemia (Harteveld and Higg, 2010). Chui *et al*., (2006) discussed the four main types of alpha thalassaemia genotypes, the deletion of one gene, which is of genotype (-α/αα), and people with this condition are referred to as 'silent carriers,' and also termed as heterozygous alpha+-thalassaemia or alpha^{+}-thalassaemia trait. These individuals are usually asymptomatic, have mild to moderate microcytic hypochromic anaemia due to compensating high red blood cell count.

The deletion of two genes has two possible genotypic presentations, where there is the deletion of one gene each from the two chromosomes (-α/-α), (homozygous alpha^{+}-thalassaemia) the other involves the deletion of the two genes from one of the two chromosomes (--/αα) (heterozygous α^{0}-thalassemia).

Carriers of α^0-thalassemia are usually asymptomatic and show microcytosis and hypochromasia (low MCV and MCH), normal percentages of HbA2 and HbF and RBC inclusion bodies (Galanello *et al*., 1998).

The three-gene deletion α-thalassaemias has a genotype of (--/-α) that produces serious haematological problems (Olivieri, 1999). Hb H generally results from the combination of one alpha$^+$-thalassaemia (-α / or α T α /) and one α^0 thalassaemia (- -/) defect. The disease presents with a moderately severe but variable anaemia, except for the rare case of α^0 thalassaemia, which combines with a non-deletional mutation of α globin gene, resulting in hydrops fetalis (Chui *et al*., 2003).

Hb H does not carry oxygen properly, making it functionally useless to the cell. Second, haemoglobin H protein damages the membrane that surrounds the red cell with the precipitation of basophilic stippling inclusion bodies thereby accelerating cell destruction (Chui *et al*., 2003, Harteveld and Higg, 2010). The combination of the very low production of alpha chains and destruction of red cells in haemoglobin H disease produces a severe, life-threatening anemia. Individuals with this form of α-thalassaemia suffer from severe anaemia, splenomegaly, bone deformities, and fatigue. Untreated, most patients die in childhood or early adolescence (Chui *et al*., 2003).

When there is a deletion of all four genes, this form of alpha-thalassaemia genotypically represented as (--/--) is deadly and the condition is also referred as hydrops fetalis syndrome.

Foetuses become anaemic early in pregnancy, hydropic (retain fluids), frequently develop enlarged hearts and livers, and most are miscarried, or die shortly after birth (Yaish, 2005).

The most common type of α-thalassaemia therefore is the deletion of one (-/α) or both (-/-) duplicated α genes (α α) on chromosome 16 (Lell *et al.,* 1999).

In people of African descent the $-\alpha^{3.7}$deletional alpha+-thalassaemia is the most predominant (Kittamis *et al.,* 1996, Mockenhaupt *et al*., 1999a). In the Northern and Ashanti regions of Ghana, there have been reports of the high prevalence of α-thalassaemia, especially heterozygous alpha+-thalassaemia affecting between 26-33% of the population (Mockenhaupt *et al.,* 2001; 2004a). Prevalence of $-\alpha^{3.7}$ alpha+-thalassaemia in the Mediterranean is about 5-10%, 20 to 30% in West Africa, and approximately 68% in the South Pacific (Yaish, 2005). Hendricks (2003) also reported that, amongst African Americans, heterozygous alpha+-thalassaemia is 30% prevalent, whereas the homozygous alpha+-thalassaemia is 2-3%. On the North Coast of Papua New Guinea (PNG), alpha+-thalassaemia is almost at fixation affecting more than 90% of the population (Allen *et al*., 1997).

Malaria reportedly accounts for 10% of Africa's disease burden (Abdul-Aziz, 2006), with more than 90% of the global morbidity and mortality of malaria suffered by Africa (Mockenhaupt *et al*., 2004c), mostly affecting children less than 5 years old.

It is the single most important cause of morbidity and mortality in Ghana, especially in children under five years, pregnant women and the poor. Clinical malaria accounted for 33.45% of all outpatient attendances and 30.3% of all hospital admissions, 34.6% of deaths in children below the age of five and 5.8% of total deaths. In these children, malaria is a leading cause of mortality, with an estimated 14,000 dying each year. Malaria is hyper endemic with year-round transmission in the northern part of Ghana with a dry season from September to April and a perceptible seasonal variation. Parasite rate ranges from 10-70% with *Plasmodium falciparum* accounting for about 90-98% of all infections, *Plasmodium malariae* for 2-9%, and *Plasmodium ovale* for 1% (National Guidelines for Laboratory Diagnosis of Malaria, MOH, Ghana 2010).

The incidence of *Plasmodium falciparum* malaria notwithstanding, discussions have been whether or not alpha+-thalassaemia individuals are protected from severe *Plasmodium falciparum* parasitaemia (Wambua, 2006; Mockenhaupt *et al.,* 2004c; Pasvol, 2006; Fowkes *et al*., 2008; Weatherall and Clegg 2010; Harteveld and Higg, 2010).

Previous studies have expressed varied opinion on the protection from severe *Plasmodium falciparum* malaria by alpha+-thalassaemia.

Initially, not only was it suggested that alpha+-thalassaemia offered no protection against severe malaria, it was even reported, that alpha+thalassaemia predisposes one to contracting mild malaria (Williams *et al*., 1996).

The incidence of contracting mild malaria and protection from either asymptomatic *Plasmodium falciparum* parasitaemia or clinical episodes of malaria were increased among alpha^{+}-thalassaemic children compared to their normal counterparts (Allen *et al.,* 1993, 1997; William *et al*., 1996; Wambua *et al*., 2006). Alpha^{+}-thalassaemia has neither been found to reduce the incidence of uncomplicated malaria, nor protect from malaria infection but rather predisposes to mild clinical malaria. The disease has however been observed to limits the progression to severe malaria anaemia (William *et al.,* 1996, 2005).

Mockenhaupt *et al*., (2001) suggested that alpha+-thalassaemia predisposed Ghanaian pregnant women to *Plasmodium malariae* infection. Individuals with the Duffy genotype *(FYA/FYB)* have also been suggested to be highly susceptible to *Plasmodium vivax* infection; however, the presence of the *FYB-33* allele or Duffy negative individuals provides selective advantage against *Plasmodium vivax* infection (Cavasini *et al*., 2007). Previous reports of molecular, immunological and cytometric evidence on Duffy blood group negative inhabitants of the Luo ethnic group and anophiline mosquito in Kenya suggests the transmission of *Plasmodium vivax* infection amongst these individuals (Ryan *et al*., 2006).

Currently, there appears to be a common understanding that alpha+ thalassaemia has an ameliorating impact on severe malaria. However, unlike the sickle cell trait, current studies have not been able to provide plausible mechanisms for the protection (Pasvol, 2006, Harteveld and Higgs, 2010).

Imrie *et al*., (2004) had observed that the higher levels of haptoglobin in the homozygous alpha+-thalassaemia individual were toxic to *Plasmodium falciparum in vitro* by removing free haemoglobin released during haemolysis (Imrie *et al*., 2006). Fowkes *et al*., (2008), in a recent study conducted in Papua New Guinea, suggested that the increased number of abnormally small erythrocytes (microcytosis) associated with homozygous alpha+-thalassaemia might be responsible for the protection against severe malarial anaemia.

Aside from this, there is an apparent disagreement between researchers as to the type of $-\alpha^{3.7}$ alpha+-thalassaemia genotype, which provides the 'ameliorating effect'.

Findings from Papua New Guinea concluded that both heterozygous and homozygous alpha+thalassaemia protect against the deadly effects of malaria though protection was more evident in subjects with homozygous alpha+-thalassaemia (Allen *et al*., 1997).

Similar studies in Tamale, Northern Ghana, indicated that alpha+-thalassaemia does protect against death from malaria; however, this was only true for heterozygous alpha+-thalassaemia. The reasons for the lack of protective effect against *Plasmodium falciparum* in the homozygous $alpha^{+}$-thalassaemia were due to its low proportion and the associated statistical limitations (Mockenhaupt *et al*., 2004a). Subsequent studies in Kenya clearly conflicted with the Ghanaian report but agreed with the findings from Papua New Guinea (Williams *et al.,* 2005b).

Another Ghanaian study, while agreeing with the ameliorating impact of alpha+-thalassaemia on malaria, however, concluded that the 'impact' was specific and driven by protection from severe anaemia, the most prevalent malaria complication (May, *et al*., 2005). The study further reported protection against other malaria complications that were accompanied by anaemia. This concept of alpha+-thalassaemia malarial anaemia specificity is supported by another study from Kenya (Wambua *et al*., 2006).

Though the subject of alpha+-thalassaemia and anaemia in Africa is still to be fully understood, a study carried out in southwest Nigeria reported that alpha+-thalassemia contributes essentially to mild anaemia, but supposedly protects against severe parasitaemia (Mockenhaupt *et al*., 1999a).

Later, Mockenhaupt *et al*., (1999b) indicated that microcytosis was significantly associated with protection from haemoglobin decrease due to *Plasmodium falciparum* and that the rate of infection was lower in microcytic than in normocytic anaemia.

In another study, Rahim, (2008) found a considerable 20% prevalence of alpha^{+}-thalassaemia in microcytic hypochromic (decrease size of red cell and haemoglobin) anaemic patients. Fowkes *et al*., (2008) corroborated this finding by indicating that red cell microcytosis was associated with homozygous alpha^{+}-thalassaemia and might be responsible for the protection against severe malarial anaemia.

However, Nyakeriga *et al*., (2004b) had earlier indicated that the incidence of clinical malaria was significantly lower among iron-deficient children who were not thalassaemic than non-iron deficient children and has also been recently reported by Kabyemela *et al*., (2008) that decreased susceptibility to *Plasmodium falciparum* infection occurred in pregnant women with iron deficiency anaemia.

1.2. TYPES OF HAEMOGLOBIN

Three types of haemoglobin (embryonic haemoglobin, foetal haemoglobin, and adult haemoglobin) are synthesized. The specific arrangements of the globin chains of these haemoglobins are under the influence of specific chromosomes. The genes for beta, delta, epsilon, and gamma chains are contained in chromosome 11 (Harmening *et al.,* 2001).

Chromosome 16 contains the genes for alpha and zeta, having two genes on the chromosome for the production of alpha chains and one gene for the production of zeta chains (Figure 1.3), (Hoffbrand *et al.,* 2004**)**. Haemoglobin has a constant two alpha chains in its configuration (Ceila, 2007) (Figure 1.1).

The β globin chain synthesis replaces the γ chain 3-6 months after birth despite its initially low levels. In the adult there are three types of haemoglobin normally present: Hb A ($\alpha_2\beta_2$), Hb A_2 ($\alpha_2\delta_2$) and Hb F ($\alpha_2\gamma_2$) (Hoffbrand *et al.,* 2004**).**

During foetal life the majority of the haemoglobin is of the Hb F type and consists of two alpha (α) chains and two gamma (γ) chains of which there are two types denoted Gγ and Aγ which are similar to β chains but differ from each other by only a single amino acid (Klug *et al.,* 2000).

During embryonic life, Hb- Gower 1($\zeta_2\varepsilon_2$), Hb- Gower 2($\alpha_2\varepsilon_2$) and Hb- Portland ($\zeta_2\gamma_2$) dominate at different stages (Hoffbrand *et al.,* 2004)(Figure 1.3).

During development from the embryo to adulthood there is a decline in gamma chains whilst beta chains increase resulting in the adult haemoglobin A (α_2 β_2), and this is in addition to small quantities of HbA_2 and Hb F (Stryer *et al*., 2002; Hoffbrand and Moss 2010)

1.2.1 Structure of haemoglobin

The haemoglobin molecule is made up of a haem portion and a globin portion. The structure of the haemoglobin molecule produces an internal environment of hydrophobic residues, which protect the iron of haem from water and from oxidation and hydrophilic external residues that render the haemoglobin molecule soluble (Harmening *et al.,* 2002).

The oxygen transport function of haem is due to the fact that the haem portion of haemoglobin, which is formed in the nucleated red cell, combines four irons in the ferrous state, is surrounded by a porphyrin ring causing the haem to combine reversibly with O_2 so that haemoglobin can function as an O_2-transporting protein (Bunn, 1993).

The iron of haemoglobin is normally in the ferrous form (Fe^{2+}) but the oxidation of iron to the ferric form (Fe^{3+}) is poorly reversible, thus converting haem to haematin and haemoglobin to methaemoglobin, that cannot transport oxygen (Klug *et al.*, 2000, Hoffbrand and Moss, 2010). The oxidation of haemoglobin to methaemoglobin and the enzymatic reconversion to haemoglobin can be determined by the Methaemoglobin Reductase Method for G6PD screening (Brewer *et al*., 1962; Bhasin and Chahal 1996)**.**

The globin portion of haemoglobin protects the haemoglobin from oxidation, renders the molecule soluble and permits variation in O_2 affinity, by 2,3-diphosphoglycerate (2,3-DPG) (Hoffbrand *et al.,* 2006).

1.2.2 Molecular basis of haemoglobin synthesis

The erythroid precursors, from the proerythroblast stage to the reticulocyte stage are the sites for haem synthesis (Ponka, 1997) with the various stages of haem synthesis taking place in the mitochondria or within the cytosol. Eight enzymatic reactions are involved, four in the mitochondrion (that is the first and the last three) and the four intermediate enzymatic reactions in the cytosol.

Luvigsen, (1998) and Hoffbrand and Moss (2004) had described the first rate-limiting step in haem synthesis as the formation of δ-aminolaevulinic acid (ALA) by condensation of glycine and succinyl CoA that is under the control of ALA-synthase with pyridoxal-5′-phosphate as cofactor (Figure 1.2).

The availability of iron affects the rate of formation of ALA; thus, iron deficiency causes iron regulatory proteins to bind to iron-responsive elements in the messenger RNA (mRNA) for ALA-synthase resulting in repression of translation.

Luvigsen, (1998) indicated that synthesis of ALA is followed by its entry into the cytosol with the formation of porphobilinogen and ALA-dehydrase influencing the coming together of the two molecules. The formation of uroporphyrinogen III is preceded by the combining effect of four molecules of porphobilinogen.

Uroporphyrinogen III is then modified in two further steps to form coproporphyrinogen III. The entry into the mitochondria by coproporphyrinogen III is followed by its conversion to protoporphyrin IX. Under the influence of ferrochelatase is the combination of ferrous (Fe2+) iron with protoporphyrin IX to form haem (also referred to as ferroprotoporphyrin) Luvigsen, (1998). The release of iron from its carrier within the endocytotic vesicle is followed by the reduction to the ferrous form, transferred to the mitochondrion for haem synthesis or stored as ferritin within the cytoplasm (Hoffbrand *et al.,* 2004). A negative feedback, control of haem synthesis by haem inhibits both ferrochelatase and the acquisition of iron from transferrin. ALA production is also inhibited by a reduced cellular uptake of iron whereas iron deficiency and erythropoietin enhance uptake of iron by erythroid cells (Klug *et al.,* 2000)

The iron-responsive element in the mRNA for the transferrin receptor protein is then protected from degradation, leading to increased expression of transferrin receptors on erythroid cell membranes and increased iron uptake.

The erythroid precursors, from the proerythroblast to the reticulocyte stage are sites for the synthesis of α and β globin (Steinberg and Adams 1991) whereas the δ chain synthesis, which ceases before the reticulocyte stage, occurs in the cytoplasm, on ribosomes (Weatherall *et al.,* 2001b).

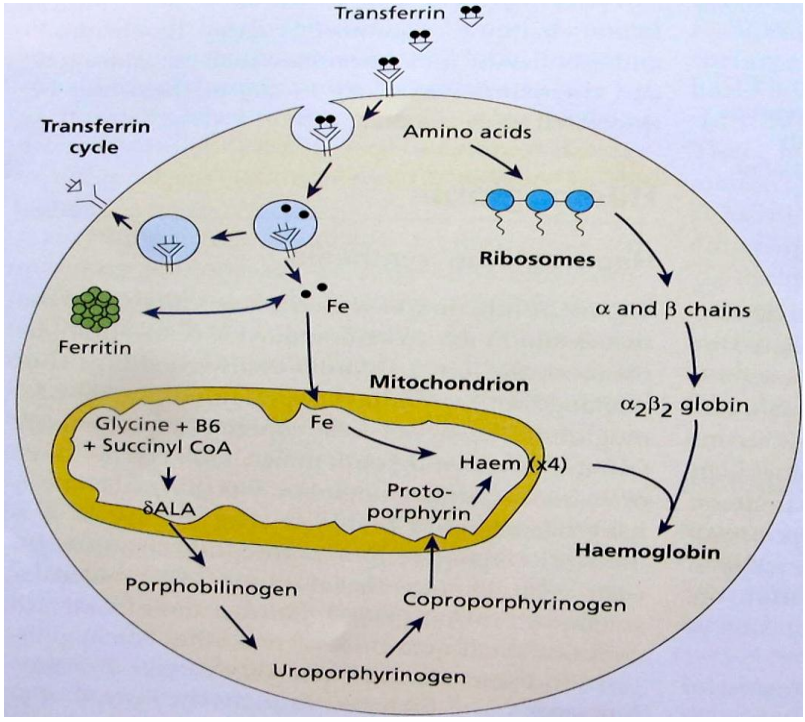

Figure 1. 2: Haemoglobin synthesis in the developing red cell. The mitochondria are the main sites of protoporphyrin synthesis, iron (Fe) is supplied from circulating transferrin; globin chains are synthesized on ribosomes. δ-ALA, δ-aminolaevulinic acid; CoA, coenzyme A (Courtesy of Hoffbrand and Moss, 2010)

The genes for globin chain synthesis are located in two clusters on chromosomes 11 and 16, with the α gene cluster close to the telomere of chromosome 16, at 16p13.3 and the β gene at 11p15.5. Also in these clusters are non-functional 'pseudo genes', which are homologues of globin genes that are not translated but are transcribed (Figure 1.3).

The α cluster of chromosome 16 extends over 28 kb and contains, in the following order, αζ gene (also referred to as ζ2), a pseudo ζ gene (ψζ or ψζ1), two pseudo α genes, (ψα2 and ψα1) and two α genes, designated α2 and α1. The β cluster on chromosome 11 contains, in the following order, and ε gene, two γ genes, designated Gγ and Aγ, respectively, a pseudo β gene (ψβ), a δ gene, and a β gene. (Stamatoyannopoulos *et al.,* 1994)

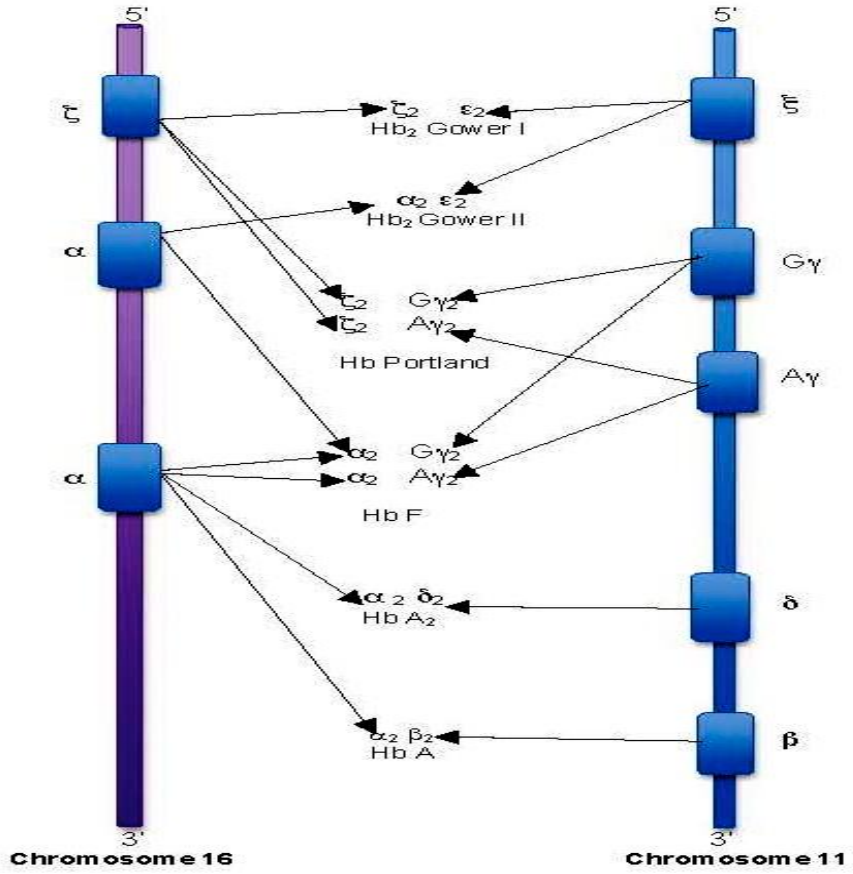

Figure 1.3: Specific chromosomes relative to human haemoglobin formation. The α cluster is located on chromosome 16 and contains, ζ gene and α2 and α1 gene. The β cluster on chromosome 11 contains the ε gene, Gγ and Aγ, δ gene, and a β gene. Reduction in the synthesis of the alpha-globin chains of adult haemoglobin (HbA, $\alpha_2\beta_2$) results in microcytosis and hypochromasia. Hb Portland is the only functional Hb and oxygen carrier in infants with Hb Bart's and Hb H (Adapted from Ciela Betty, 2007)

All human globin genes have three coding sequences (exons) and two intervening non-coding sequences (introns), which are flanked by 5', and 3' non-coding sequences (untranslated regions, UTRs) (Nagel and Jaffé 2001). Alpha-globin genes are duplicated haemoglobin-alpha locus 1 (HBA1) and haemoglobin-alpha locus 2 (HBA2). HBA1 is the gene encoding for α_1-globin and HBA2 codes for α_2. These two genes are associated with α-thalassaemia. As normal allelic variants, both HBA1 and HBA2 have three coding exons. The mRNAs produced by HBA1 and HBA2 have identical coding regions and can be distinguished only by their 3' UTR, however as pathologic allelic variant, the deletion of one or both HBA1 and HBA2 is the most common cause of α-thalassaemia (Galanello *et al*., 1998). The expression of HBA1 and HBA2 is regulated by a region known as the hypersensitivity site (HS–40) located 40kb upstream from the α-globin cluster. The deletion of HS-40 results in an α-thalassaemia phenotype, inspite of the structural integrity of both α-globin genes (Higgs, 2001).

1.2.3 Normal haemoglobin

Normal adult haemoglobin is made up of haemoglobin (Hb) A, HbA_2 and F. Hb A_2 comprises 2-3.5% of total haemoglobin with a rise in adult, but lower at birth (0.2-0.3%) (Serjeant *et al.,* 1978).

Its functional properties are similar to Hb A, but inhibit the polymerization of Hb S (sickle cell haemoglobin) by making the HbSS RBCs more deformable thus preventing microvascular occlusions and haemolytic anaemia characteristic of the disease, (Steinberg and Adams, 1991), and have higher affinity for oxygen and pan-cellular distribution.

The slower rate of δ chain synthesis compared to β chain is the result of reduced rate of synthesis of Hb A_2.

Absolute or functional iron deficiency anaemia (IDA), α, δ and $\delta\beta$ thalassaemia trait cause a proportional reduction in Hb A_2 (Nagel and Steinberg 2001), however the proportion of Hb A_2 is increased in patients with β thalassaemia trait and some patients with unstable haemoglobin.

Hb F is increased during intrauterine life with a very high oxygen affinity but weak affinity for 2,3-DPG, sex, inherited characteristics both linked and unlinked to the β gene clusters but vary from 60% to 90% at birth and appear slightly higher in women (Jane compared to Hb A. This is important in facilitating the transfer of oxygen from the mother to the foetus (Bunn and Forget, 1986). The percentage of HbF is determined by age and (Cunningham, 1998).

1.2.4 Abnormalities of haemoglobin

Normal adult haemoglobin (HbA) comprises $\alpha_2\beta_2$ but also contains small quantities of HbF ($\alpha_2\gamma_2$) and HbA_2 ($\alpha_2\delta_2$).

Primarily, the function of normal haemoglobin is the carriage of oxygen from the lungs to the tissues (Hoffbrand *et al.*, (2010). Because of the more stable protein of the normal haemoglobin it is able to convert to a coloured pigment called cyanmethaemoglobin, and this conversion forms the basis for most of the colorimetric procedure used to measure haemoglobin (Ceila, (2007). However abnormal haemoglobins are toxic and have higher oxygen affinity. For example methaemoglobin oxidises iron to Fe^{3+}, which cannot bind oxygen and individuals become cyanotic, whilst carboxyhaemoglobin has affinity for carbon monoxide and causes carbon monoxide poisoning. The exposure to sulphonamides or sulfa-containing drugs results in the formation of sullfhaemoglobin (Bunn, 1993). In as much as abnormal haemoglobins could result from accidental or purposeful ingestion or absorption of substances and drugs they could also result from inherited defects.

These abnormalities could be due to the synthesis of abnormal haemoglobin (haemoglobinopathies) and/ or reduced rate of synthesis of α or β - globin chain (thalassaemia) (Brown, 2003). A number of variant haemoglobins results from point mutations some of which the new codon does not code for amino acid (nonsense mutation); because of their termination of chain synthesis, their proximity to the 3^{I} end of the gene results in the production of an abnormal but functional globin chain. If proximal a short but unstable chain is produced leading to a beta thalassaemia phenotype (Nagel and Steinberg, 2001).

Hoffbrand *et al.*, (2010), observed that most of the haemoglobinopathies are clinically silent; but sickle cell anaemia as well as thalassaemia major are the clinically most important abnormality. However, haemoglobin C, D and E are also common and, like Hb S occur as a result of substitutions in the β chain.

According to Hoffbrand *et al.*, (2010), unstable haemoglobins are rare and cause chronic haemolytic anaemia of varying severity with intravascular haemolysis; It has been observed that the genetic effects such as sickle cell offer protection against *Plasmodium falciparum* malaria in the heterozygotes (Aidoo *et al.,* 2002) hence the high prevalence of the heterozygote in malaria endemic areas.

1.3 THE THALASSAEMIAS

The name thalassemia comes from the Greek words “thalas” meaning sea and “emia” which stands for blood. Generally, thalassaemias are prevalent in populations that evolved in humid climates where malaria is endemic (Tassiopoulos *et al.,* 2005).

The thalassaemias are an increasing public health problem worldwide with over 750 different mutations that can be identified, mostly affecting either alpha-chain (alpha thalassaemias) or β chain (beta thalassaemia) (Vichinsky, *et al.,* 2005). The frequency of the thalassaemias appears to be high in developing countries (Weatherall, 2008).

In many of these countries, however, facilities for the control of the disease are limited and the situation is compounded by the unwillingness of governments accepting that the thalassaemias present a diagnostic burden that may lead to inappropriate iron therapy (Weatherall, 2010).

In most African countries, and for that matter Ghana, the more detailed frequency and economic data, which are required to provide solid evidence for the health burden posed by the thalassaemia, is non-existent. In addition many people may be carrying the disease but do not know they carry it.

The thalassaemias are the most common single gene disorders in the world involving inherited disorders of haemoglobin synthesis that result from an alteration in the rate of globin chain production, (Murray, *et al.,* 2003, Modell and Darlison, 2008, Colah *et al*., 2010)**.** The disorder results in decrease and defective production of haemoglobin, with estimates of gene frequencies ranging from 2.5% to 15% in some areas of the tropics (Modell and Darlisson, 2008). A reduction in the rate of production of certain of these globin chain/chains hinders the synthesis of haemoglobin and creates an imbalance with the other normally produced globin chains (Nagel and Steinberg, 2001). As a direct result of this imbalance between normally produced and the under-produced globin chain, an excess of the normally produced type accumulates in the cells as an unstable product and begins reacting with cell membranes, hindering normal cellular processes and acting as foreign particles, leading to the destruction of red cells (Brown, 2003).

The degree of destruction or toxicity varies according to the type of excess chain involved with the type of thalassemia normally going by the name of the under-produced chain/chains (Yaish, 2005).

The reduction in the chain production could vary from a slight decrease to a complete absence of production. A superscript symbol indicates whether a subunit is completely absent (α^0 or β^0) or whether its synthesis is reduced (α^+ or β^+) (Maggio *et al*., 2002). Of the four possible types of thalassaemias, alpha, and beta thalassemias are the most prevalent resulting from gene deletion or beta point mutations in the non-coding portion of the globin gene.

Beta (β) thalassemia results when there is reduction in β-chains production (β+) or when there is no production of β-chain (β^0) and alpha (α) thalassemia refers to reduction in the production of α-chains but also due to the deletion of α-chains of haemoglobin (Chui *et al*., 2006).

Olivieri (1999) discussed the deficiency in these chains and indicated abnormalities in the formation, size, and shape of red blood cells (RBCs) resulting from ineffective production of RBCs and their destruction. The heterogeneity and complex nature of this inherited disease involves clinical manifestations such as ineffective haematopoiesis, and accelerated haemolysis due to abnormal haemoglobin synthesis (Haen, 1993).

The severity of the above clinical manifestations is dependent on the number of globin chain(s) deleted.

As a result, people with thalassaemia often have a reduced number of RBCs, which can affect the transportation of oxygen to body tissues (Allen *et al*., 1997). However in the carrier states a normal or increased number of RBC is observed due to compensated process. In addition, thalassaemia can cause RBCs to be smaller (microcytosis) resulting in reduced mean cell volume (MCV)

As a result of the compensating increased RBC found in carriers of α+ and α^0 thalassaemia alleles, they present with mild to no anaemia and therefore require no treatment. However, there appears to be no cure for Hb Bart's Hydrops Foetalis Syndrome and the HbH disease. (Harteveld and Higgs 2010) The best treatment available today consists of frequent blood transfusions (every two to three weeks) with iron chelation therapy (e.g. deferoxamine) administered subcutaneously (Allen *et al*., 1997). Bone marrow transplants (hematopoietic stem cell transplantations) and cord blood transplantation with pre-operative myeloablation are potentially curative (Tassiopoulos *et al*., 2005).

1.3.1 Epidemiology

The alpha thalassaemias also follow a pattern of distribution along the tropics where malaria is highly prevalent. The alpha thalassaemia afford their carriers some protection against death from *Plasmodium falciparum* infection by depriving the parasites of nutrients normally derived from digestion of haemoglobin (Cheesbrough, 2005).

Other researchers have also suggested that parasitized alpha thalassaemic cells bind more antibody than normal cells, thus facilitating the clearance of malaria parasites from the blood (Teo and Wong 1985; Luzzi *et al.,* 1991).

Mockenhaupt *et al.*, (2000) reported that alpha thalassaemia and beta thalassaemia affect as many as 33% and 1% respectively, of the antenatal population in a Ghanaian community. The same study demonstrated that alpha thalassaemia trait protects the carriers from malaria. The prevalence of alpha^{+}-thalassaemic trait in Tamale, Ghana and its surroundings was estimated to be 31.8% (Mockenhaupt *et al.*, 2004a).

The protective effect of thalassaemia trait against severe malaria was also demonstrated. The protection however, was shown to wane with age (>5 years) with the explanation that innate resistance acts mainly before specific immunity has developed. However, studies in the Melanesia (William *et al.,* 1996) have shown that children homozygous for alpha^{+}-thalassaemia who were less than 5 years had more than twice the incidence of severe malaria.

In Tanzania, Enevold *et al*., (2008) observed reduced risk of *Plasmodium falciparum* in children older than 5 years and suggested that the increase susceptibility of subjects who were less than 5 years old might result in improve immunity to subsequent severe disease with an enhanced antigen expression on *Plasmodium falciparum*-infected alpha^{+}-thalassaemia erythrocytes.

1.3.2 Beta (β)- Thalassaemia

This group of inherited disorders is characterized by impaired synthesis of beta (β) - globins chain(s). Beta globin genes (located on chromosome 11), are inherited in pairs – one from each parent.

The β thalassaemia is common in regions bordering the Mediterranean Sea (Southern Italy, Sardinia, Greece, Sicily, Turkey, Lebanon and Armania), Arab, African Americans, Asia and Africa (Haen, 1993)**.**

Affected individuals exhibit a variable reduction in β globins chain synthesis ranging from a minimal deficit (mild β^+ thalassaemia alleles) to complete absence (β° thalassaemia), which are caused by point mutations that affect the β globins gene locus and are extremely heterogeneous (Forget 2001) (Table 1.1)**.** The consequences of this mutation therefore are the reduction in total red cell haemoglobin in the β-thalassemia major due to the precipitation of excess α chains in the erythroblast and in mature red blood cells leading to ineffective erythropoiesis and haemolysis (Hoffbrand *et al.*, 2010) This damage to red blood cell precursors in the marrow and ineffective erythropoiesis caused by intracellular denaturation and precipitation of unmatched alpha chains results in the formation of inclusion bodies (Babior and Stossel, 1994). The reduction in total red cell is however corrected to some extent by mopping up of excess α chains by the production of γ chains, the synthesis of haemoglobins containing other β - class chains (Hb F and Hb A_2), but fail to compensate fully for the decreased quantity of Hb A in thalassaemia cells.

Pallister, (1994) described three groups of β thalassaemia as β thalassaemia minor (trait), β thalassaemia major and β thalassaemia intermedia of which the mildest form, β thalassaemia minor arises from the inheritance of a single abnormal β globin gene. Individuals who inherit this single β thalassaemia allele, either β° or β^{+} have thalassaemia trait.

Moi *et al.,* (2004) have described the β° or β^{+} thalassaemia trait as being clinically asymptomatic but with a mild anaemia and characteristic hypochromic microcytic red blood cells, elevated levels of Hb A_2 ($\alpha_2\delta_2$) and variable increases of Hb F ($\alpha_2\gamma_2$). Thein, (2004) describe β° thalassaemia as characterized by the complete absence of Hb A ($\alpha_2\beta_2$) as no β chain or small amounts of β+ is synthesised and results from the inheritance of two β° thalassaemia alleles (homozygous or compound heterozygous states).

This condition, β thalassaemia major will normally present with profound anaemia requiring regular blood transfusion without which the patient may die within the first 2 years of life. Inheritance of two β thalassaemia alleles, however, does not always lead to thalassaemia major and many patients with two β thalassaemia alleles have a milder disease, ranging from a condition that is slightly less severe than transfusion-dependence to one that is asymptomatic and often mistaken for β thalassaemia trait (Thein, 2001).

The diverse collection of phenotypes between the two extremes of β thalassaemia major and β thalassaemia trait constitute the clinical syndrome of β thalassaemia intermedia (Moi, *et al.,* 2004).

Table 1.1: Types of β- Thalassaemia

Type	Defect in Globin Chain Production
β^+ - thalassaemia	Diminished β- chain
β^0 – thalassaemia	No β- chain
δβ- thalassaemia	No β or δ- chain
Hb Lepore	No β- chains, δ- chain replaced by δβ- hybrid
Hereditary persistence of foetal Hb	No β- or δ- chain but increased γ- chain production

(Adapted from Babior and Stossel 1994)

1.3.3 Alpha (α)-Thalassaemias

Normally four α globin genes of which two copies each located on chromosome 16 regulate α globin synthesis. Each individual receives four α-globin genes from their parents – a maternal pair and a paternal pair (Hoffbrand *et al*., 2006). The production of alpha chains is divided among the four α-globin genes. Thus, each individual has four genes for the production of alpha chains (Ceila, 2007). The alpha globin chains produced by these four genes, along with the appropriate amount of beta globin chains then associate with the haem molecule to form the normal adult hemoglobin (Weatherall and Clegg, 2001b). Alpha thalassaemias refer to the under-production of α-globin chains.

Alpha thalassaemia results when one or more of these genes fails to function properly and with the amount of α-globin produced falling short of that required the normally produced beta globin chains will pair with the available, but limited, α-chains with leftover beta chains accumulating in the cellular environment (Old, 2003)**.** Though dysfunctional, rather than completely deleted genes are occasionally found, the defect in α-thalassaemia usually involves the total deletion of one or more of the genes due to deletion or mutation. Since the α chain is a component of both foetal and adult haemoglobins, the α-thalassaemia is especially severe (Leung *et al*., 2005). In West Africa, the mutant genes responsible for alpha+ thalassaemia are reported to be 20 to 30% prevalent (Mockenhaupt *et al.*, 1999a).

Some works within the Sub-region are in agreement with this level of prevalence, though some figures seem to slightly exceed it (May *et al.*, 2007; Mockenhaupt *et al.*, 2004a; and Falusi *et al.*, 1987). Apart from Africa, other regions of the world also have high prevalence and in Southeast Asia and Papua New Guinea, the prevalence is about 2 times that of Africa's. Alpha thalassaemia is known to equally affect both sexes. Ethnically, though, the disease is diverse, with different mutations (i.e. $-\alpha^{3.7}$ and $-\alpha^{4.2}$) being more prevalent in some regions than others, and at times even being unique to others (Yaish, 2005).

1.3.4 Types of alpha thalassaemia

As indicated earlier, each individual inherits two sets of alpha globin genes from their parents. A normal individual is of the genotype (αα/αα), showing that all the α-globin genes are present and functioning properly.

The types of alpha thalassaemias depend on how many of these genes are deleted or dysfunctional, and in rare cases, having a mutation (Ohene-Frempong *et al*., 1980, Weatherall 2001a, Leung*, et al.,* 2005). (Figure 1.5)

1.3.5 The molecular basis of alpha+-thalassaemias

The two main genes associated with alpha- thalassaemia are HBA2 (the gene encoding α2) globin and HBA1 (the gene encoding α1 globin). These regulate the normal individual alpha globin synthesis (Galanello *et al*., 1998). However the expression of these genes is dependent on a Multispecies Conserved Sequences (MCS-R2) or the erythroid-specific DNAsel hypersensitivity sites (HS-40) located 40 kb upstream from the alpha globin clusters which have been shown to express alpha globin genotype (Harteveld and Higgs 2010) and appears to be the major regulator. The deletion of MCS-R2 (HS-40) therefore results in an alpha-thalassaemia phenotype.

The molecular basis of alpha+ thalassaemia is the deletions that remove one of the linked pairs of α-globin genes, leaving the other one intact (–α/αα).

However, there are situations where both α-globin genes are intact with one having a mutation that either partially or completely inactivates it (αTα/αα) (Weatherall 2001b).

1.3.6 Deletion of a single alpha globin gene (alpha+-thalassaemia mutation)

Each α- globin gene lies within a boundary of homology of 4 kb long generated by duplication, subsequently subdivided by insertions and deletions, resulting in three homologous sub segments that are designated X, Y and Z (Pallister, 1994) (Figure 1.4)

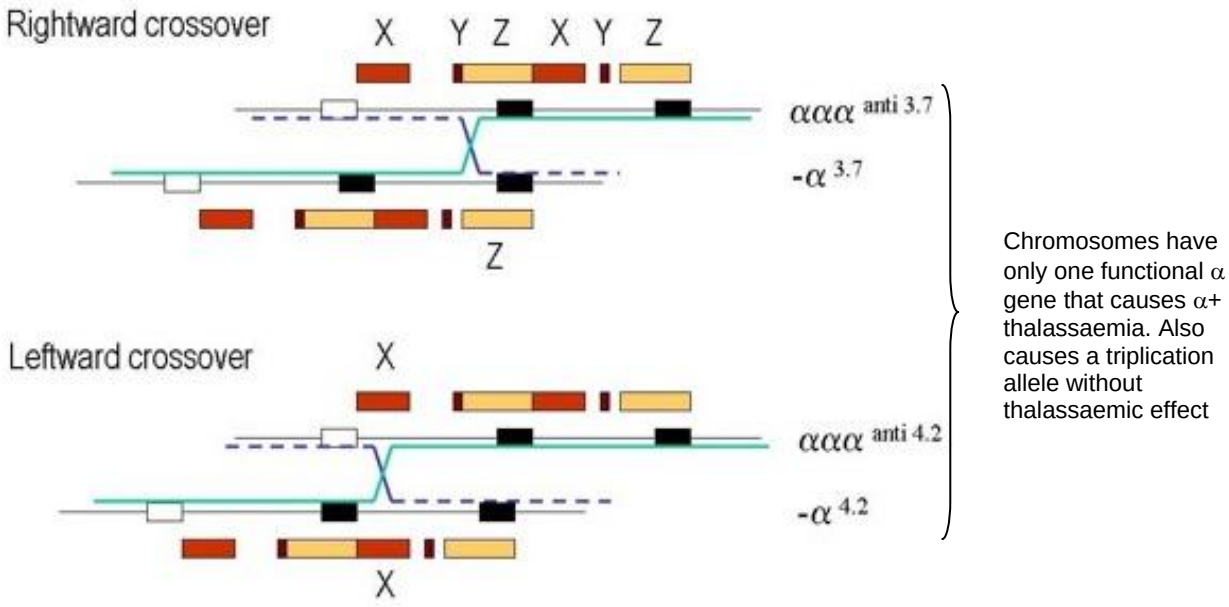

Figure 1.4 Deletions resulting in alpha+- thalassaemia mutations (Adapted from Harteveld and Higgs 2010)

The duplicated Z box homology is 3.7 kb apart and the X box homology is 4.2 kb apart. Misalignment and unequal crossover between these segments at meiosis produces two chromosomes 16 with either a single (–α) or triplicated (ααα) α-globin genes.

Chromosomes with three α genes represent the reciprocal $\alpha\alpha\alpha^{anti\ 3.7}$ and $\alpha\alpha\alpha^{anti\ 4.2}$ formed during the process of unequal crossing-over Pallister (1994). If the reciprocal recombination occurs between the Z boxes, which are 3.7 kb apart, a 3.7 kb of DNA is lost, an event that is described as a *rightward deletion* ($-\alpha^{3.7}$). A crossover between the two X boxes which are 4.2 kb apart, results in the deletion of a 4.2 kb of DNA, and referred to as the *leftward deletion* ($-\alpha^{4.2}$) (Steinberg, 2001, Harteveld and Higgs 2010)

1.3.6.1 Deletion of one gene on one or both chromosomes

This type of alpha thalassaemia results from the deletion of all parts or part of the α2-globin gene. It is of genotype (-α/αα) is actually of no haematological consequence because the production of α-globin proteins is only slightly altered since only one gene is deleted (Bain, 2006, Hughes-Jones *et al.,* 2009)(Figure 1.5)

It is so close to the normal condition that it is very difficult to detect without the use of specialized laboratory equipment and techniques. People with this condition are referred to as 'silent carriers.' The condition is also termed as heterozygous alpha^{+}-thalassaemia or alpha^{+}-thalassaemia trait (Wintrobe and Lee, 1999) and the most common mutations causing this disease are the $-\alpha^{3.7}$ and $-\alpha^{4.2}$. There is also the situation where there is the deletion or inactivation of two alpha-globin chains in trans configuration (-α/-α) (Figure 1.5). This is termed as homozygous alpha+ -thalassaemia (Higgs, 2001)

1.3.7 Deletion of both alpha globin genes on one chromosome (alpha^0-thalassaemia mutation)

The deletions, which remove both alpha globin genes in *cis* renders these chromosomes unable to synthesize any alpha chains (Galanello *et al*., 1998). These mutations range from approximately 6 kb to more than 300 kb. The most common types of the alpha^0-thalassaemia mutations are the Southeast Asian (- -^{SEA}), Filipino (-- ^{FIL}), and Mediterranean (--^{MED}) (Harteveld and Higgs 2010) and in the homozygous state, neither the embryonic ($\zeta_2\gamma_2$) nor the foetal ($\alpha_2\gamma_2$) haemoglobins could be made with these mutations result in Hb Bart Hydrops Fetalis Syndrome. However, if any of the alleles occur in combination with another allele carrying a single deletion like the –α$^{3.7}$, the outcome is Hb H disease (Higgs, 2001)

1.3.7.1 Deletion of two genes

The type of thalassaemia where there is the deletion of the two genes from one of the two chromosomes (--/αα) (Figure 1.5). (Chui *et al*., 2006) and is termed as heterozygous α^0-thalassaemia. The heterozygous α^0-thalassaemia genotype is a determinant of both haemoglobin H disease and haemoglobin Bart's disease (Johnston, 2005). The two-gene deletion alpha-thalassaemia, at times referred to as alpha-thalassaemia trait or mild alpha thalassaemia, is characterized by at most mild anaemia and small RBCs, though some other patients experience no symptoms.

Most heterozygous α^0-thalassaemias do not present with any important clinical or haematological differences between the different mutations giving rise to α^0-thalassaemia genotype (Bain, 2006, Hughes-Jones *et al.,* 2009).

1.3.7.2 Deletion of three genes

Three-gene deletion α-thalassaemias, with a genotype of (--/-α) (Figure 1.5), produce serious haematological problems. The most common causes are compound heterozygosity for α^+-thalassaemia and α^0-thalassaemia or alternatively compound heterozygosity for α^0-thalassaemia and a non-deletional α-thalassaemia (Bain, 2006, Hughes-Jones *et al.,* 2009). Individuals with this form of α-thalassaemia suffer from severe anaemia, splenomegaly, bone deformities, and fatigue (Bain, 2006).

Since only one of the alpha globin genes is producing alpha globin proteins, there are very few alpha proteins to pair with the beta proteins, which, are still being produced normally. Due to this imbalance, the surplus beta proteins begin to associate in groups of four (β^4), producing the abnormal haemoglobin referred to as haemoglobin H. (Johnston, 2005, Chui *et al*., 2006). There are two main problems with HbH. First, it is unable to carry oxygen properly, thereby rendering it useless to the cells. Second, Hb H proteins damage the red blood cell membrane, thereby speeding up cellular destruction (Weatherall 2010). Because of the severe anaemia, patients with this disease usually require blood transfusions to survive.

1.3.7.3 Deletion of all four genes

This form of alpha-thalassaemia, genotypically represented as (--/--),(Figure 1.5), is the most deadly of all α-thalassemias. This condition is said to be 'incompatible' with life. The condition, also referred as Hydrops Fetalis Syndrome, is characterized by the deletion of all four of the α-globin genes, meaning that there is no production of alpha proteins (Harteveld and Higgs 2010, Hughes-Jones *et al.,* 2009).

By extension, this means that absolutely no haemoglobin A (normal adult hemoglobin) or haemoglobin F (fetal hemoglobin) is produced (Chui *et al*., 2006). Because of the lack of alpha proteins, the gamma proteins, which are produced during fetal life and under normal circumstances should have paired with the α-proteins, begin to associate in groups of four ($\gamma 4$). This association forms the abnormal haemoglobin, called haemoglobin Bart's (Hb Bart's) (Johnston, 2005). Fetuses that are affected by Hydrops Fetalis Syndrome become anaemic early in pregnancy.

Additionally, they become hydropic (retain fluids), frequently develop enlarged hearts and livers, and most are miscarried, stillborn, or die shortly after birth.

However, there are reports that a few of such babies have survived, but only after intrauterine blood transfusion (Yaish, 2005).

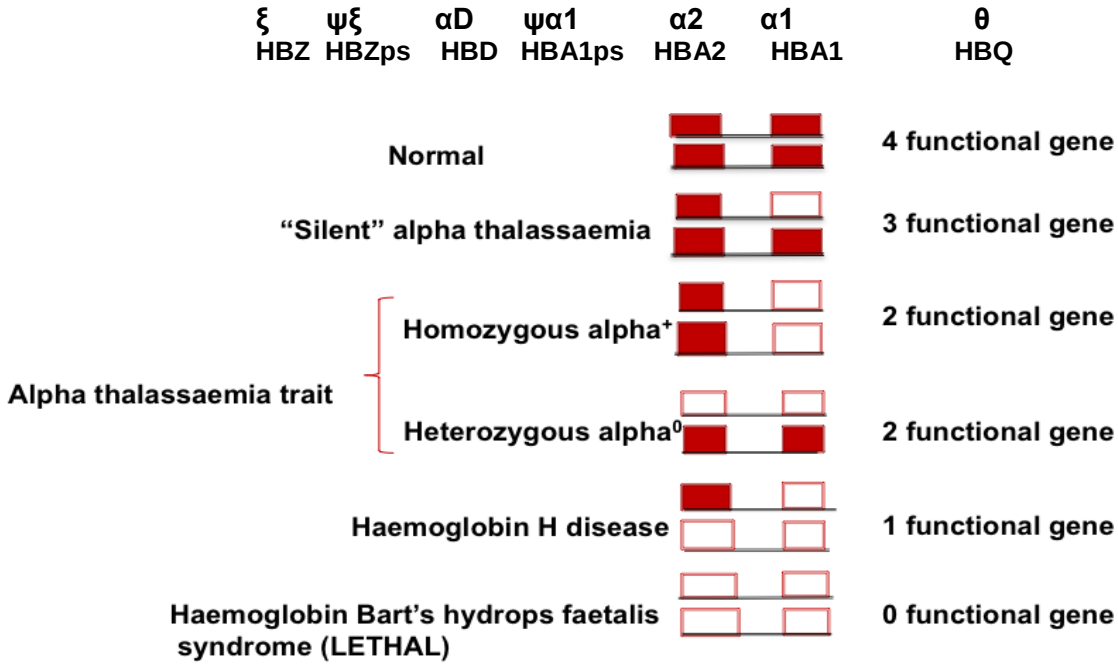

Figure 1.5: Classification of the deletion of alpha globin gene and phenotypical expression –Deletion of alpha globin gene

1.3.8 Non deletional alpha-thalassaemia

There are situations where both α-globin genes are intact but point mutation in the HBA2 (the gene encoding α2) globin (αTα) or the HBA1 (the gene encoding α1) globin (ααT) genes either partially or completely inactivates expression from a chromosome (Galanello *et al*., 1998). This results in non-deletion forms of alpha thalassaemia. These non-deletional forms of alpha thalassaemia mainly occur in HBA2

The non-deletional forms of α-thalassaemias have been described where these conditions have been observed to present with a more severe reduction in α chain synthesis than the deletional types (Harteveld and Higgs 2010).

The most common ones are either due to polyadenylation site mutation, termination codon mutations or structural mutations resulting in α-globin variant that is unstable (Table 1.2). Alpha^{0} thalassaemia is rare among people of African decent but alpha+ thalassaemia though of little clinical significance; homozygous for the more severe non-deletional α^{+}-thalassaemias ($\alpha T\alpha$/ $\alpha T\alpha$) may have the clinical features of haemoglobin H disease (Bain, 2006).

One of the most common forms of non-deletional alpha thalassaemia caused by a mutation of the termination codon of the α2-globin gene is Haemoglobin Constant Spring (-- $^{SEA}/\alpha$ ^{CS} α). Impaired mRNA translation consequent on initiation codon of α2-globin gene produces non-deletional mutations among Africans (Table 1.2).

Examples of such non-deletional mutations are haemoglobin Seal Rock and haemoglobin Evanston (Harteveld and Higgs 2010, Bain, 2006). Among Southeast Asians who are carriers of Haemoglobin Constant Spring, the prevalence is about 8% (Vichinsky, 2009). In the Middle East, the common alpha thalassaemia non-deletional mutation is Haemoglobin α $^{TSaudi}\alpha$. It is a mutation of the polyadenylation signal sequence of the α2 gene, resulting in decrease expression of structurally normal α chains. Haemoglobin Koya Dora, another structural non-deletional mutation, is found in India. Other structural mutations such as haemoglobin Quong Sze found in Southeast Asia are highly unstable and result in defect in the haem pocket (Vichinsky, 2009).

1.3.9: Other forms of alpha+-thalassaemia deletions

Some rare deletions affecting the α-globin genes have been associated with abnormalities usually affecting intellectual capabilities of the affected α-thalassaemic patient (Higgs and Weatherall 2009). These are the α-thalassaemia mental retardation syndrome on chromosome 16 (ATR16), the α-thalassaemia mental retardation X-linked syndrome (ATR-X) and α-thalassaemia myelodysplastic syndrome (ATMDS). In these conditions, deletions remove not only the α globin genes but also a number of other genes around them (Harteveld and Higgs 2010)

Table 1.2 Classification of non-deletional mutations causing alpha-thalassaemia in Africans

Affected sequence	Affected gene	Mutation(s)	HGVS	Synonym Hb -name	Distribution	Phenotype
mRNA translation						
Initiation codon	- $\alpha^{3.7}$	init ATG>GTG	c.1A>G p.Met1Val		African	α^0
	- $\alpha^{3.7\ II}$	init (-2 bp)	c.-2_-3delAC		N-African, Med	α^+ - α^0
Exon I	α2	Cd22 (-C)	c.69delC p.Gly23fs		African	
	α2	Cd23 (G>T)	c.70G>T p.Glu24X		Tunesian	α^0
	- α	Cd30/31(-2 bp)	c.94_95delAG		African	α^0
Exon II	α1	Cd62(-G)	c.187delG p.Val63fs		African	
Exon III	α2	Cd116 G>T	c.349G>T p.Glu117X		African	α+
	α2	Term Cd TAA>GAA	c.427T>G p.X143Glu	Seal Rock	African	α+
Post translational						
Exon I	- α	Cd14 T>G	c.43T>G p.Trp15Gly	Evanston	African	α+

Note- del-deletion; Cd-codon; term-termination codon; init-initiation codon; HGVS-Human genome variation society. (Adapted from Harteveld and Higgs (2010)

1.4.0 Distribution of alpha+-thalassaemia

Weatherall, (2001a) discussed the global distribution of α-thalassaemia and observed that the alpha+-thalassaemia provides a selective advantage in regions that are endemic for *Plasmodium falciparum* malaria. He further indicted that the protection might be due to thalassaemic red blood cells providing poor environment for the malaria parasite so that carriers are less likely to develop severe malaria and die from it (Weatherall, 2001a)

Alpha+-thalassaemia is predominant in the tropics and sub-tropics, with its prevalence ranging between 1% and 98% (Higgs *et al.*, 1989).

However, in the wake of increase in global migration with consequent inter-racial marriages, there has been a resultant upsurge in the worldwide occurrence of the disease (Liu *et al.*, 1999). Alpha^{+}-thalassemia occurs commonly across tropical Africa, the Middle East, certain regions of India, and throughout Southeast Asia. The highest frequencies occur in the Pacific Island populations (Vichinsky, 2000).

With the advent of DNA technology, it has become possible to genotype α-thalassemia, a previously difficult task (Weatherall and Clegg, 1999). The benefit of this breakthrough is that we now have an appreciable level of understanding as to which forms of α-thalassemia are most prevalent in which parts of the world. Though α-thalassemia has been found to occur in all races and ethnic groups, certain genotypes are more common in certain ethnic groups and regions than in others (Yaish, 2005, Leung *et al.,* 2005) (Table 1.3)

For instance, α^{0}-thalassemia is limited in its distribution to the Mediterranean regions and parts of South East Asia, especially Southern China, Thailand, and Vietnam (Weatherall and Clegg, 2001b). This explains why haemoglobin H disease and hydrops fetalis syndrome are most prevalent in Asia or people of Asian decent.

In Africa, and in people of African descent around the world, the $-\alpha^{3.7}$ deletional alpha+-thalassaemia is the most predominant (Kattamis *et al.*, 1996, Old, 2003).

In support of this, a study conducted in Nigeria, Africa's most populous nation, found a 27% prevalence of the $-\alpha^{3.7}$ alpha+ thalassemia in the South-western part of the country (Mockenhaupt *et al.*, 1999b).

Table 1. 3 The prevalence of alpha^{+} and alpha^{o} thalassaemia in different ethnic groups and their clinical severity

GENE	ETHNIC GROUP	SEVERITY	PREVALENCE
Heterozygous alpha^{+}-thalassaemia. Silent carrier or mild alpha thalassaemia minor; alpha^{+}-thalassaemia trait. (-α/αα)	Africa	Patients are asymptomatic but may be slightly anaemic.	**North Africa 5-8%(Egypt 8%); Gambia 8-55%; Togo 46%; Nigeria 8-58%; Senegal 22%; Benin and Burkina Faso 29%; Ivory Coast 39%; Kenya 19-34%, Tanzania 2% CAR 39%(23% of pygmies); Republic of Congo 36-40% (29% of pygmies); Zambia 20-27%; Malawi 39%; Namibia 11.5%; South Africa (coloured-7%, black 12%-36%); Mozambique 5-6%, Madagascar<1-3%; Comoros 2%; Africans in UK 25-30%; African-Americans 25%; Afro-Caribbeans 25% (Jamaica 34%); Ghana 26-33%**
Homozygous alpha^{+}-thalassaemia (-α/-α)	Africa, African American Melanesia, Polynesia	No significant symptoms of disease. Mild microcytic hypochromic anaemia. Absence of splenomegaly	Same as above
Heterozygous alpha^{0}-thalassaemia(--/αα)	Mediterranean, South-East Asia, Southern China, India, Vietnam, Thailand	No significant symptoms of disease. Mild microcytic hypochromic anaemia	Greece 1.5%; Cyprus 2%;
Haemoglobin H Disease alpha^{o} alpha^{+} double heterozygous (--/ -α)	Mediterranean, South-East Asia, Southern China,	Splenomegaly in most patients. Ineffective erythropoiesis. Defective Hb synthesis and haemolytic component. Ineffective O_2 transport by Hb H. Iron depletion. Marrow shows erythroid hyperplasia	Rare
Haemoglobin Bart's Hydrops fetalis or homozygous α^{o} thalassaemia (--/--)	South-East Asia, Mediterranean region	No functional Hb formed. Intrauterine death followed by stillbirth usually occurs between 25 and 40 weeks of gestation, or the baby dies very shortly after birth.	Rare

Adapted from Kattamis *et al.*, (1996); Weatherall and Clegg, (2001); Yaish, (2005)

In Ghana, the picture reflects the general situation on the continent, especially the West African sub-region. The most prevalent form of α-thalassemia in Ghana is the alpha+-thalassemia trait, both the heterozygous (-α/αα) and the homozygous (-α/-α). According to Timmann *et al.,* (2007), the Ashanti region of the country has a moderate prevalence of the $–α^{3.7}$ deletional alpha thalassemia (Figure 1.6). A number of studies done in different parts of the country all seem to be in agreement with the 20 to 30% prevalence rate of alpha+-thalassemia in West Africa (Borges, *et al,* 2001), though there are some slight variations (Mockenhaupt *et al.*, 2001; Mockenhaupt *et al.*, 2004a; May *et al.,* 2007).

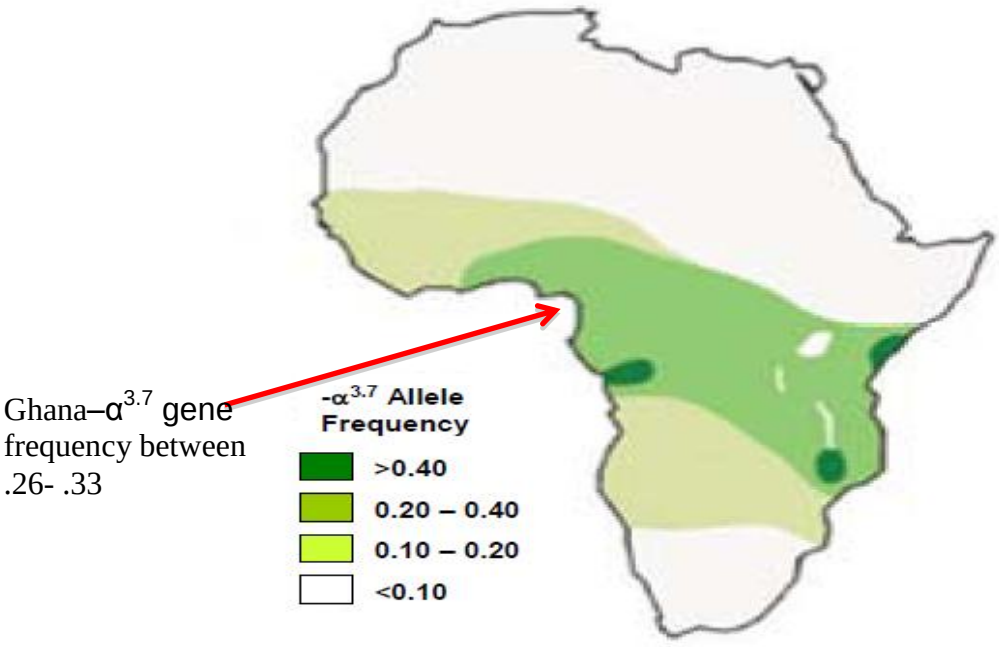

Figure 1.6: Allele Frequency of $-α^{3.7}$ within Africa (Adapted from Wintrobe and Lee 1999).

1.4.1 Diagnosis of alpha-thalassaemia

The International Committee for Standardization in Haematology expert panel on abnormal haemoglobins and thalassemias made diagnostic recommendations regarding laboratory investigation. Baseline tests that were recommended include a complete blood count (CBC), electrophoresis at alkaline pH, tests for solubility and sickling, and quantification of Hb A_2 and Hb F (Clarke and Higgins, 2000). If an abnormal Hb is identified on the preliminary tests, then further techniques were recommended to identify the variants. These techniques include electrophoresis at pH 6.0–6.2, globin chain separation, and isoelectric focusing (IEF), HPLC and DNA analysis (Clarke and Higgins, 2000)

Other information essential when diagnosing alpha+-thalassemia include race, ethnic background, family history, and age (Yaish, 2005). Trent, (2006) indicated a careful three tier approach involving: (1) full blood count (2) special haematological tests, followed by (3) DNA mutation analysis, which may provide the most effective way to detect primary gene mutations as well as gene-gene interactions that can influence the overall phenotype. For those with red cell microcytosis (MCV < 76 fL), a reliable measure of iron status is required (e.g. serum ferritin) to exclude iron deficiency (Vulliamy 2001).

The production of alpha globin proteins starts early in foetal life, which therefore means that α-thalassaemia (Hb Bart's Hydrops Foetalis), can be diagnosed even at the earliest stages of human development.

Diagnostic tests at the foetal stage are either by chorionic villus sampling or amniotic fluid testing (Galanello *et al.,* 1998 and Vijay *et al.,* 2010). Some forms of the disease, like homozygous alpha^{+}-thalassemia and heterozygous alpha^{0}-thalassemia are of no serious haematological consequences, except during stressful events like illness or pregnancy when some sufferers have mild anaemia (Hoffbrand and Moss 2010). Other more deadly forms of the disease like non-deletional Hb H disease or Hb Bart's disease result in life-threatening situations. Some signs and symptoms to look for in diagnosing α-thalassemia include slow growth, pallor, jaundice, and splenomegaly, hepatomegaly, and bone deformities.

1.4.1.1 Complete Blood Count (CBC)

The key components of the complete blood count (CBC) include: haemoglobin value (Hb), red blood cell (RBC) count, mean cell volume (MCV), mean corpuscular haemoglobin (MCH) and red cell distribution width (RDW) (Bertram 1991). Whilst red cell indices are observed as critical to the diagnosis of thalassemias, structural haemoglobinopathies may have an impact on the red cell indices (Lafferty *et al.,* 1996). Rahim (2008) indicated that initial haematological data presents the key to successful detection and characterization of the haemoglobinopathies, particularly the thalassaemias. The red cell indices are important indicators in the assessment of thalassaemia trait, which is characterized by microcytosis (Bergstrome and Poon, 2002).

MCV can be considered as a key diagnostic indicator. Low MCV (mean corpuscular volume) or MCH (mean corpuscular haemoglobin) might provide a clue for α-thalassaemia especially in regions at risk, and iron deficiency must be excluded as a first step (Wonke *et al.,* 2007). However MCH value of < 26.5 pg have been suggested to be a sensitive indicator of thalassaemia trait (Savitree *et al*., 2009). The RBC count is useful as a differential tool because the thalassemias produce a microcytic anaemia that is associated with increased RBC count, while the other causes of microcytic anaemia, including iron deficiency and anaemia of chronic disease, are more associated with a decrease in the RBC count that is proportional to the degree of decrease in Hb concentration (Clarke and Higgins, 2000).

The Hb concentration can provide complementary information since carriers of alpha thalassemia do not frequently have reduced Hb.

1.4.1.2 Microcytosis

When haemoglobin synthesis is retarded and an extra mitotic division occurs before the erythroblast dies mature red cell which are smaller than normal are formed which are known as microcytes. Microcytosis is a blood disorder characterized by the presence of microcytes; often associated with anaemia in which the average size of erythrocytes is smaller than normal (Pallister, 1994).

Microcytic anaemias are due to defects in haemoglobin biosynthesis resulting from reduced supply of iron (iron deficiency), inadequate delivery of iron (anaemia of chronic inflammation), impaired production of haem (sideroblastic anaemia), or defective synthesis of the globin component of haemoglobin (thalassaemia) (Old, 2003). Microcytosis may be due to iron deficiency but also heterozygous or homozygous alpha^{+}-thalassaemia or alpha^{0} thalassaemia trait and β-thalassaemia (Figure 1.8) (Wonke *et al.,* 2007; Eivazi-Ziaei *et al*., 2008). The presence of microcytosis in carriers for alpha thalassaemia or beta thalassaemia has been suggested as a reflection of an increased number of terminal cells divisions during erythropoiesis, due to the combination of defective haemoglobinization of the erythrocytes and a highly proliferative bone marrow (Fowkes *et al*., 2008). The general classification of thalassemias however, is hypochromic and microcytic anaemias (Figure 1.7).

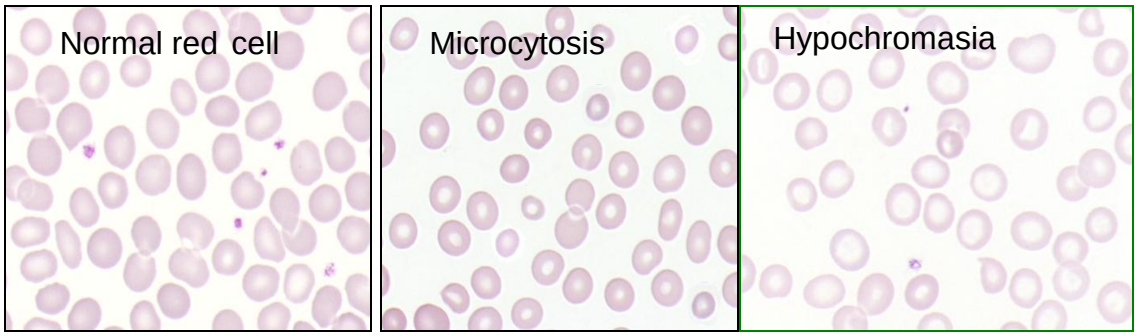

Figure 1.7 Red cell morphology showing normal red cells, microcytosis and hypochromasia x 40

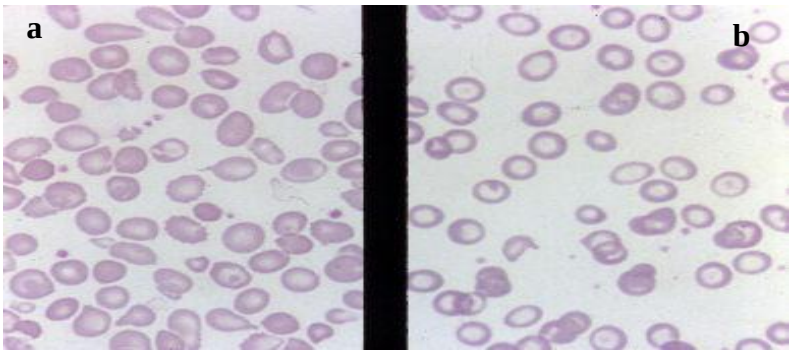

Figure 1.8: Red Blood Cell Morphology showing alpha thalassaemia (a), and iron deficiency (b) x 40

1.4.1.3 Iron studies

It is essential that iron deficiency be excluded before any further tests are undertaken in haemoglobinopathy workups. All samples that showed microcytosis are further evaluated for plasma or serum ferritin, serum iron, and iron binding capacity, percentage saturation levels and transferrin receptor levels (Table 1.4) to exclude iron deficiency, leaving probable alpha+ thalassaemia as causes of microcytosis in those with normal ferritin (Trent, 2006, Wonke *et al*., 2007). Iron deficiency (ID) and malaria are the major causes of anaemia in children in sub-Saharan Africa. Diagnosis of iron deficiency appears unreliable in the presence of malaria and other inflammatory conditions. This is because in areas of high malaria transmission and infection it is difficult to distinguish states when cellular iron delivery to the erythroblast is decreased even though iron stores are adequate (termed functional iron deficiency) from situations where limited amount of iron is due to lack of available iron in the reticular endothelial system (Phiri *et al.,* 2009). However, plasma ferritin level at a cutoff of <273 μg/L have been suggested to provide a 79% specificity and 79% sensitivity and best predicts iron deficiency anaemia (IDA) (Phiri *et al.,* 2009).

Ong *et al*., (2008) had also indicated that in populations with a high prevalence of thalassaemia trait, the serum transferrin receptor (sTfR) level might not be useful in diagnosing iron deficiency unless the patient's thalassaemia status is known. He further explained that raised sTfR level is unaffected by inflammation and therefore useful as a marker of iron deficiency. However, diseases that cause an increase in erythropoietic activity can also result in a raised sTfR level. Thalassaemia trait is associated with ineffective erythropoiesis and therefore increases sTfR level (Ong *et al*., 2008)

Table 1.4: Haematological parameters in alpha thalassaemia and other haematological conditions

	Iron Deficiency	**Chronic Inflammation or Malignancy**	**Alpha-Thalassaemia**	**Sideroblastic Anaemia**
MCV, MCH	Reduced in relation to severity of anaemia	Normal or mild reduction	Reduced; very low for degree of anaemia	Usually low in congenital type but MCV often raised in acquired type
Serum Iron	Reduced	Reduced	Normal	Raised
TIBC	Raised	Reduced	Normal	Normal
Serum Transferrin Receptor	Raised	Normal or low	Variable	Normal
Serum Ferritin	Reduced	Normal or raised	Normal	Raised
Bone Marrow Iron Stores	Absent	Present	Present	Present

(MCV; mean corpuscular volume, MCH; mean corpuscular haemoglobin, TIBC; total iron- binding capacity Courtesy of Hoffbrand, *et al,* (2004)

1.4.1.4 Haemoglobinopathy evaluation

1.4.1.4.1 Haemoglobin Electrophoresis

This test measures the type and relative amounts of haemoglobins present in the red blood cells. It is the most common test for the initial detection and characterization of variant haemoglobin (Bain, 2006). In principle, haemoglobins are able to separate from each and the bands visualised when applied to a membrane and exposed to a charged gradient.

The choice of methodology will usually depend on the volume of workload, whether sample is liquid blood or dried blood spots, the ease of handling, reproducibility, local availability of expertise and cost (Ryan *et al*., 2010).

1.4.1.4.2 Cellulose acetate electrophoresis (CAE) at alkaline pH

This method permits the initial identification of haemoglobins A, F, S/G/D/ Lepore, C/E/O-Arab, H at pH of 8.2 to 8.6. The consumable for CAE are less expensive however the method is time consuming and labour cost may be relatively high (Bryan *et al.,* 2010). Cellulose acetate electrophoresis relies on charge change for separation and in general gives similar mobility to IEF, although IEF does show better resolution than CAE. Scanning densitometry or elution followed by spectrophotometry could be used to quantify normal or variant haemoglobin on cellulose acetate (Clarke and Higgins, 2000).

However because of the electrophoretic similarity of many structurally different types of haemoglobin, the evaluation must be supplemented by other procedures such as acid (agarose) gel, HPLC or IEF (Bain, 2006).

In the presence of microcytosis, a single band may represent compound heterozygous for variant haemoglobin or β^0 thalassaemia (Ryan *et al*., 2010).

A negative sickle-screening test in children less than six months of age should be viewed with caution. Alkaline or acid haemoglobin electrophoresis or high performance liquid chromatography (HPLC) or isoelectric focusing (IEF), or a combination of these, should be used to assess the sickle status in children less than six months of age. This is due partly to the smaller amount of Hb S present and partly to the larger amount of Hb F in newborns (Stephens, 2005)

1.4.1.4.3 isoelectric focusing (IEF)

Isoelectric focusing can be used as an alternative to CAE at alkaline pH, but not as an alternative to acid agarose or citrate agar. It can however be used to complement these tests (Stephens, 2005). Although time consuming and labour intensive, it has an excellent resolution than CAE and has been used to identify and quantify haemoglobins (Clarke and Higgins, 2000). Citrate agar (and acid agarose) relies not only on charge but also on solubility of the different haemoglobins in agar. It provides a good separation of Hb F from Hb A and haemoglobins S, C, D-Punjab, E and O-Arab (Stephens, 2005).

1.4.1.4.4 High Performance Liquid Chromatography (HPLC)

The Bio-Rad Variant quantifies HbA2, HbF, HbA, HbS and HbC. HPLC is suitable for use in the diagnosis of β-thalassaemia trait because it provides an accurate quantification of HbA2. A greater percentage of HbA_2 and/or HbF are usually seen in beta thalassaemia trait but are reduced in alpha thalassaemia (Clarke *et al.,* 2000). In severe iron deficiency anaemia, Hb A2 may be lower (by up to 0.5%) and if the Hb A2 is borderline, or 3.0–3.5%, the iron deficiency is corrected then repeating the Hb A2 before making a final decision about whether or not an individual is a carrier for ß-thalassaemia (Stephens, 2005). Derivatives or post-translational adjuncts of HbS co-elute with HbA2 and HbA^0 rendering its quantification inaccurate. In the homozygous for HbS this can lead to falsely raised HbA2 and presence of HbA, that could result in patient wrongly diagnosed as HbSβ+thalassaemia (Stephens, 2005, Ryan *et al*., 2010).

A reduction in Hb A_2 level is significant in patients with Hb H disease. However a more sensitive method is to identify inclusion bodies in the red blood cells by staining peripheral blood cell with 1% Brilliant Cresyl Blue (Harteveld and Higgs 2010). These inclusion bodies may also be found in carriers of homozygous $alpha^0$ genotypes and carriers of non-deletional alpha+ -thalassaemias.

In this study, the haemoglobinopathy evaluation was used to exclude HbAS trait and HbAC individuals, as they are known to protect from the effect of severe malaria.

They are serious confounders in epidemiological studies of alpha+-thalassaemia in Africa and needed to be excluded (William *et al*., 2005a, 2005c, Wambua *et al*., 2006)

1.3.9.5 DNA analysis

DNA analysis of each deletion breakpoint is carried out using either Southern blot hybridization or single-tube-multiplex-Polymerase Chain Reactions (PCR) techniques. This method has been developed to detect the two most common deletional alpha+-thalassaemia that is, $-\alpha^{3.7}$ and $-\alpha^{4.2}$ and the 5 alpha^{0} – thalassaemia deletions which are the $-\alpha^{20.5}$, Southeast Asian (- $-^{SEA}$), Filipino (-- ^{FIL}), Mediterranean (--^{MED}) and Thailand (--^{Thai}). The -PCR-based testing is more rapid, less expensive, safer (no radioactivity involved), more sensitive, and easier to interpret than Southern blot analysis (Bowden *et al*., 1992, Dode *et al*., 1993, Baysal & Huisman, 1994, Oron-Jarni *et al*., 1998). For various reasons, this test has not found its way into routine use but can be used to diagnose thalassaemia, and to determine carrier status. It is the only reliable way of diagnosing carriers who have only one of four alpha genes deleted or mutated. The single-tube multiplex PCR assay is used for the frequently observed determinants of alpha^{+}–thalassemia that allows simple, high throughput genetic screening for these common haematological disorders (Chong *et al*., 2000). The PCR uses primers that anneal to the component single strands of the target DNA to amplify the DNA sequences for the investigation of deletions and mutations in the alpha and beta globins.

This is followed by enzymatic extension of the primers (from their 3' ends) using a thermostable DNA polymerase (Chong *et al*., 2000). PCR therefore selectively amplifies specific target genomic DNA and rapidly isolates DNA whose structure is already partly sequenced and detect small amounts of one specific type of DNA (Liu *et al.,* 1999, 2000). Sequence analysis can be used to identify point mutations (including the rare termination codon mutations and hyperunstable α-globin variants) in the coding regions of HBA1 (α1) and HBA2 (α2) when an alpha-globin deletion is not identified and alpha-thalassaemia is highly suspected (Traeger-Synodinos *et al*., 2000)

1.5 MALARIA

Malaria is a parasitic disease caused by five Plasmodium species – *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, and Plasmodium malariae and Plasmodium knowlesi.* Four of the about 170 Plasmodium species are known to cause malaria in humans (Mayo Foundation for Medical Education and Research, (MFMER), 2007). The most prevalent and by far the most virulent of the four is *Plasmodium falciparum*. The known vector of these parasites is the female *Anopheles* mosquito. The male however feeds on nectars (Kwiatkowski, 2005). The primary mode of infection is when a parasite-carrying mosquito bites an uninfected person and, in the process of taking its blood meal, injects the parasites into the person's blood (Snow *et al*., 2005).

Other modes of transmission include blood transfusion and from-mother-to-child transmission or congenital malaria (Cheesbrough, 2005).

1.5.1 Life cycle of the *Plasmodium falciparum* parasite

Infection of the human host occurs when a female *Anopheles* mosquito injects sporozoites during a blood meal into the dermis of the host. The major phases of the life cycle are: liver stage, blood stage, sexual stage, and sporogony (Snow *et al*., 2005) (Figure 1.9). The sporozoites migrate to the liver, where they develop into thousands of merozoites. The matured merozoites enter the bloodstream invading the RBCs; develop into the ring stage trophozoite, followed by a mature trophozoite, and finally, the schizont (Miller *et al.,* 2002).

The mature schizont gives rise to approximately 16 daughter merozoites, which are released upon the rupture of the RBCs (Umar *et al*., 2007; Nyakeriga *et al*., 2004a) starting the clinical disease presentation of fever (>37.5^{O}C), chills, headache, fatigue, seizures, and coma, primarily due to dysregulation of the cytokine response (Umar *et al*., 2007; Nyakeriga *et al*., 2004a,). The sexual stage of the life cycle of the parasite commences when another female *Anopheles* mosquito ingests a gametocyte during a blood meal. Within the mosquito's gut where the temperature is lower, male and female gametocytes emerge from the ingested parasitized RBCs to produce a zygote also known as the ookinete. The ookinete forms an oocyte that contains immature sporozoites at mid-gut of the mosquito.

These sporozoites travel to the mosquitoes' salivary glands and are ingested into the human host to start a new life cycle (MRPMRC, 2005)

1.5.2 Malaria Transmission

Malaria transmission differs in strength and regularity depending on local factors such as rainfall patterns, proximity of mosquito breeding sites, and the mosquito species (WHO 2007). These factors combined, explain to a certain extent, why Africa, especially Sub-Saharan Africa, with its tropical and subtropical climate, is the most widely affected.

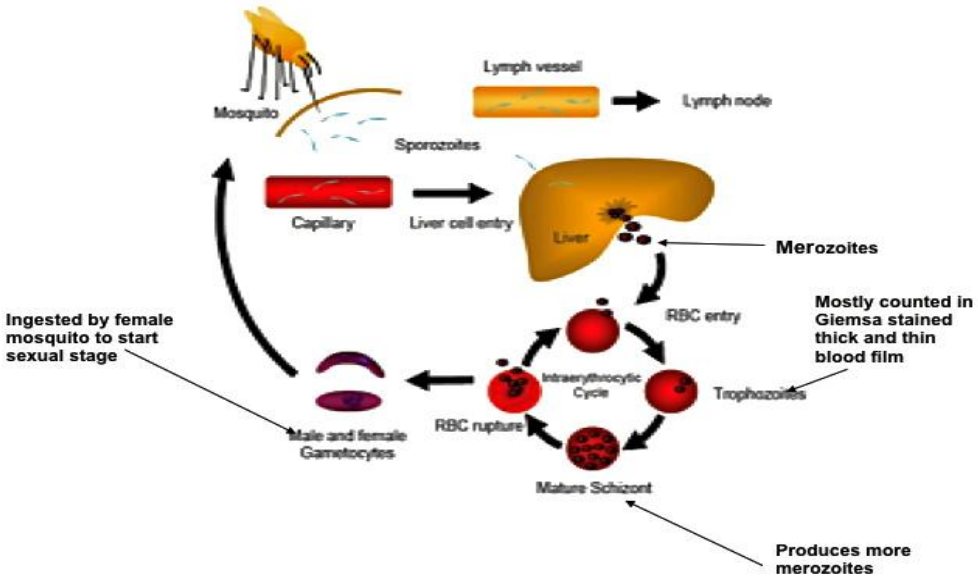

Figure 1.9: Life Cycle of *Plasmodium falciparum* in Humans (Adapted from Snow *et al.,* 2005)

Malaria has had far-reaching ramifications on the continent of Africa. Apart from the severe death toll, it also has serious economic implications for the continent's developing countries. Malaria is said to slow economic growth in affected countries by approximately 1.3% per year (WHO, 2007). The direct and indirect cost of malaria in Africa is estimated at $ 12 billion per year (Abdul-Aziz, 2006).

The finding that malaria-affected families are able to harvest only 40% of their crops compared with healthy families (MRPMRC, 2005), gives meaning to this high economic cost, as farming/agriculture is one of the major sources of income for most African nations. Considering these harsh statistics, there appears to be a clear link between malaria and poverty. According to Abdul-Aziz (2006), approximately 60% of all malaria cases occur among the poorest 20% of the world's population. This apparent link establishes grounds for waging a concerted campaign against this disease, because this would not only halt the disease, but also ease, to a certain extent, the current economic problems.

1.5.3 National Interventions

Globally however, deaths from malaria have reduced from an estimated 350 million to 250 million per year due to the Roll Back Malaria Programme under the Millennium Development Goals (MDGs).

For children under 5 years in Africa, malaria deaths dropped from an estimated 3000 to 2000 children per day due to household distribution and ownership of insecticide-treated bed nets (ITN), from 3% in 2000 to 42% in 2010 (Ministry of Health (MOH), Ghana 2011). It is estimated that malaria accounts for 22 % of under five years mortality and 9 percent of maternal deaths (Roll back malaria, 2005).

In 1999, Ghana adopted the Roll Back Malaria initiative and has since been implementing a combination of curative and preventive interventions.

Children less than five years of age and pregnant women are targeted for the distribution of ITNs (Ghana Demographic and Health Survey 2008)

Some countries in Africa have achieved more than 50% reduction (Ghana has achieved about 37.5%) in either confirmed cases of malaria or malaria admissions and deaths over the past decade.

In the past 10 years 736,700 children in 34 African countries have been saved from malaria through the use of ITN, indoor residual spraying (IRS), effective medicine, and preventive treatment during pregnancy. The Ghana Health Service provides sulphadoxine-pyrimethamine to pregnant women as intermittent preventive treatment (IPT) against malaria free of charge and as directly observed therapy (DOT) at both public and private antenatal services (Roll back malaria, 2005)

The adoption of Artesunate-Amodiaquine, an Artemisinin-based Combination Therapy (ACT) as the drug of choice for the treatment of uncomplicated malaria was implemented across the country.

These include two alternative ACT drugs, namely Artemether-Lumefantrine and Dihydroartemisinin-Piperaquine for those who remain hypersensitive to Artesunate-Amodiaquine (UNICEF, 2007).

The Ghana Health Service also conducts information, education, and communication (IEC) activities on these malaria control interventions, using a variety of communication media and strategies.

None the less, malaria still remains hyper-endemic in Ghana accounting for about 61 percent of hospital admissions of children under five years and 8 percent of admissions of pregnant women (UNDP 2000). About 3 to 3.5 million cases of suspected malaria are reported each year in public health facilities, representing 30-40 % of outpatient attendance (Ministry of Health (MOH) Ghana 2011). Of this figure, over 900,000 are children under the age of five.

1.5.4 *Plasmodium falciparum* malaria mortality in Ghana

Present reports indicate that malaria incidence in urban areas is increasing rapidly. A study done in some communities of the two largest cities in Ghana, Accra and Kumasi, established a prevalence range for malaria at between 2% to 33% (Klinkenberg *et al.,* 2006). One of such reports pointed out that malaria presently presents a leading cause of morbidity and mortality among urban African populations (Donnelly *et al*., 2005). Given the current rapid growth rate of 3.5% of urban populations in Africa, about 50% of the African population will live in urban areas by 2025 (UNPP, 2005).

The incidence of malaria cases in urban settlements could possibly increase proportionally in the absence of robust combative initiatives.

Sadly, though, despite the concerted efforts, of local governments and international bodies, malaria still seems to be gaining ground.

Some regions of the world, mostly within Africa, are still recording extremely high numbers of malaria cases (Asante *et al*., 2003). A study in 2001 involving 530 pregnant women in Ghana revealed a 63% prevalence of *Plasmodium falciparum* alone, and a further 3.4% and 2.6% prevalence of *Plasmodium malariae* and *Plasmodium ovale* respectively (Mockenhaupt *et al*., 2001). It would be logical to think that, with the current degree of awareness, the situation should have improved, but that appears not to be the prevailing reality.

In 2006, over 16 million people were infected with malaria in Tanzania, and about 100,000 dying as a result, (Abdul-Aziz, 2006). Some of the reasons cited for this escalating number of cases include drug and insecticide resistance, climate change, failure of health delivery systems and armed conflicts

1.6 ERYTHROCYTE POLYMORPHISMS AND THE BASIS FOR MALARIA PROTECTIVE EFFECT

It is recognised that some genetic factors, which could be lethal in their homozygous state, in their heterozygous state, may diminish the risk of an exposed person developing parasitaemia, the risk of a parasitaemic person of becoming ill with malaria fever, and the risk of a person with malaria fever developing severe malaria (Kwiatkowski, 2005).

1.6.1 The Duffy antigen and protection from *Plasmodium falciparum*

Innate resistance to malaria infection is conferred by some blood group polymorphism. The Duffy antigen encodes the Duffy Antigen Receptor Cytokine (DARC/Fy) chemokine receptor that is expressed on the erythrocyte, which the *Plasmodium vivax* merozoites require for erythrocyte invasion (Min-Oo and Gros 2005). A large percentage of the African population, as well as many American blacks, are Duffy negative. These individuals lack the receptor on their red blood cells due to the polymorphism in the promoter gene GATA1 resulting in reduced receptor expression and therefore impair entry of *Plasmodium vivax* with the expression of the gene blocked in developing erythrocytes by a mutation of the erythroid transcription factor (Tournamille *et al.*, 1995, Robert *et al*., 2004). However, some evidence of transmission of *Plasmodium vivax* among Duffy antigen negative individuals has been reported in Kenya (Ryan *et al*., 2006).

Other reported cases of individuals with the *FYA/FYB* genotypes showing higher susceptibility to malaria have also been observed in Brazil where the presence of FYB-33 allele is suggested to provide selective advantage in the population by reducing the rate of infection by *Plasmodium vivax* (Cavasini *et al*., 2007).

1.6.2 Erythrocyte cytoskeleton and protection from *Plasmodium falciparum*

Erythrocyte cytoskeleton changes can also affect the outcome of infection where ovalocytic erythrocytes for instance, are thought to have reduced susceptibility to parasite invasion (Genton *et al*., 1995; Mgone *et al*., 1996 and Allen *et al*., 1999). This may be due to altered binding of Southeast Asia Ovalocytosis (SAO) Band 3 to parts of the cytoskeleton leading to decreased cell deformability or limited redistribution of the protein that is observed when the parasite tries to invade. (Evans and Wellems, 2002).

1.6.3 Glucose-6-phosphate dehydrogenase (G6PD) and protection from *Plasmodium falciparum*

G6PD deficiency, globally the most common human enzymeopathy, is present in nearly 400 million individuals, resulting in decreased activity of a metabolic pathway that protects the interior of the red blood cell from oxidant stress (Ruwende and Hill, 1998). Its distribution correlates with malaria endemicity in Africa, Asia, the Middle East, and the Mediterranean.

Mutations in the gene are varied between populations with epidemiological studies supporting associations between malaria resistance and deficiency of the enzyme (Mehta *et al.,* 2000)**.**

Evidence from some studies has suggested protective effects from clinical malaria by G6PD deficiency (Aidoo *et al*., 2002).

G6PD deficiency, notably the widespread form in Africa (G6PD A-) has been indicated to confer protection against malaria in male but not female children, who have just one abnormal gene (Guindo *et al.,* 2007) but also provide protection in pregnancy of heterozygous deficient women (Mockenhaupt *et al*., 2003). Guindo and his co-workers further indicated that the protection was more evident against the life threatening cerebral malaria.

However, reported work at the Kintampo Health Research Centre in Ghana, suggested that G6PD deficient children of less than 5 years were not protected against clinical malaria (Personal communication). The mechanism by which G6PD deficiency protects against malaria attracts varied opinions. Toxic oxidized substances in the red blood cell have been suggested to inhibit parasite multiplication or alternatively, infected red blood cells may be more susceptible to phagocytosis or haemolysis because of increased cytolytic compounds and membrane damage (Cappadoro *et al.*, 1998).

Miller *et al*., (1984) and Golenser *et al.,* (1988) reported that the growth of *Plasmodium falciparum* might be inhibited more consistently under conditions of oxidative stress resulting in extensive haemolysis in G6PD deficient individuals.

However, Cappadoro *et al*., (1998) argued that the inhibition of parasite growth observed in G6PD-deficient individuals was a result of the susceptibility of their red blood cells to phagocytosis by peripheral blood monocytes, particularly at the ring-stage of infection accompanying the increase binding of IgG and complement C3 to infected red cells.

Schwarzer *et al*., (1992) had reported that G6PD deficient cells were more prone to changes in the cell membrane because of low levels of reduced glutathione (GSH) and higher vulnerability to oxidants.

1.6.4 Sickle cell trait (Hb AS) and protection from *Plasmodium falciparum*

Of the 300,000 annual births of sickle cell disease (Hb SS) globally, about 70% occurs in sub- Saharan Africa where recent reports suggest that 50% to 80% of affected children die annually (Makani *et al*., 2010). Sickle cell disease has been described as a double–edged sword with evidence suggesting that those with the sickle cell trait (HbAS) are protected against malaria whilst those with HbSS not only suffer severe health problems but also generally die from malaria (McAuley *et al*., 2010).

Previous evidence from study by Aluoch (1997) supports the idea that individuals heterozygous for the sickle-cell allele (sickle-trait; HbAS) are protected against the severe effects of malaria due to an early and enhanced acquisition of protective immunity against malaria. Aidoo *et al.,* (2002) also reported that the sickle cell trait provides significant protection against all-cause mortality, severe malarial anaemia and high-density parasitaemia.

The homozygous sickle-cell condition (HbSS) is, however, reported to be responsible for the deadly effects of sickle-cell anaemia, whereas the heterozygous condition is usually asymptomatic. According to Ayi *et al*., (2004) the high frequency of the sickle cell haemoglobin (HbS) in populations where *Plasmodium falciparum* malaria is endemic is as a result of the reduced level of the malaria parasitaemia in the mutated allele (HbA / HbS). The protection has been attributed to defective parasite growth or to enhanced removal of the parasitized RBCs.

Cheesbrough, (2005) described a “balanced polymorphism” in which death caused by *Plasmodium falciparum* malaria to the homozygotes sickle cell individual is offset by the survival advantage of heterozygotes in malaria endemic areas.

1.6.5 Haemoglobin C and protection from *Plasmodium falciparum*

Another common erythrocyte variant found in the hyperendemic malaria region of West Africa is HbC (Hb C; β6Glu→Lys), and the mutation presents with mild haematological symptoms in the homozygous (Robert *et al.,* 2004).

The prevalence of HbC reaches 40-50 % in West Africa (Burkina Faso, Ivory Coast, Ghana, in Togo and Benin (20%), the Caribbean (3.5 %) in the USA (3 %), and in North Africa in Morocco and Algeria) (1 to 10 %) (Bachir and Galacteros, 2004). Recent studies in West Africa suggest that resistance to *Plasmodium falciparum* has maintained the relatively high frequencies of HbC with evidence suggesting both heterozygote and homozygote resistance to malaria (Weatherall and Clegg, 2002).

HbC has been reported to protect from severe malaria but does not prevent infection. HbC occurs in polymorphic frequencies almost exclusively in the northern savannas of West Africa (Mockenhaupt *et al*., 2004b). Verra *et al*., (2009) indicated that HbC individuals have low *Plasmodium falciparum* invasion rate and parasite growth, reduced cytoadherence and red cell rosetting. Further indications from *in vivo* studies were that HbC individuals showed reduced uncomplicated and severe malaria. However, HbAC individuals in some ethnic groups did not show significant evidence of reduced rates of severe *Plasmodium falciparum* malaria compared to others (Agarwal *et al.,* 2000).

Whilst HbAC was observed to reduce the progression from uncomplicated malaria to severe malaria, HbC conferred protection from the severe manifestation of malaria, with a risk reduction of 80% of developing severe malaria observed amongst the Dogon people of Bandiagara, Mali, in West Africa, who were carrying HbC (Agarwal *et al*., 2000).

The situation was different in Burkinabes where HbAC and HbCC protect against clinical *Plasmodium falciparum* malaria (Modiano *et al*., 2001). Hb C was associated with a 29% risk reduction in clinical malaria in HbAC and 93% in Hb CC (Modiano *et al*., 2001) suggesting therefore a protective effect against *Plasmodium falciparum* for heterozygous and homozygous HbC in certain populations of West Africa. The findings further suggested that HbC individuals were not protected from either infection or uncomplicated malaria, an observation that is corroborated by a recent study on the differing effect of HbC on uncomplicated *Plasmodium falciparum* malaria in Ghana which indicated children with HbC were not protected against malaria (Kreuels *et al.,* 2010). However, Kreuels *et al.,* (2010) observed that the clustering of erythrocyte band 3 protein and abnormal *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), expression in HbAC erythrocytes, may prime recognition by antibodies and lead to reduced cytoadherence and enhanced elimination of parasitized red cells. This evidence for different protective effects from HbC suggests the possibility that population-specific factors, including differences in genetic background, may have a strong influence on the distributions of haemoglobinopathies in malarious regions.

A case control study in the northern part of Ghana where this study was conducted, whilst suggesting a 47% reduction of acquiring severe malaria in HbAC individuals observed that the haemoglobin polymorphism did not only prevent infection but also reduced the odds of developing severe malaria (Mockenhaupt *et al*., 2004b). The geometric parasite density (GMPD) was lower in Hb AC than was observed in HbCC and HbSC.

1.6.6 Haemoglobin E and protection from *Plasmodium falciparum*

The most common form of the variant β-globin gene (HBB; OMIM 141900) usually found in malaria endemic regions of Southeast Asia is haemoglobin E (HbE; 26Glu->Lys), which causes the HbE disease and HbE trait (Naka *et al.,* 2008). It is distributed throughout much of Southeast Asia and has gene frequencies of approximately between 13-50% in Thailand (Hutagalung, *et al.*, 1999, Fucharoen *et al*., 1998). It has been suggested that the positive selection against *Plasmodium falciparum* has been responsible for the rather high population frequency of HbE in the region (Ohashi *et al*., 2004).

Malaria complications are less severe in HbE trait, suggesting that the course of acute *Plasmodium falciparum* malaria is significantly ameliorated (Hutagalung *et al.,* 1999) due to inhibition of parasite growth in HbE red cells (Chotivanich *et al.,* 2002).

1.7 ASSOCIATION BETWEEN ALPHA^{+}- THALASSAEMIA AND *PLASMODIUM FALCIPARUM* MALARIA

The severe manifestation of *Plasmodium falciparum* is a major cause of death amongst children in particularly Sub-Saharan Africa who are below the age of five years. Alpha^{+}-thalassaemias are the commonest genetic disorders affecting humans, a reflection of the protection it confers against death from *Plasmodium falciparum* (Mockenhaupt *et al*., 2004a; and Williams *et al*., 2005b). The fact that red blood polymorphisms are evidently predominant in regions of the world where malaria reaches endemic proportions informed the suggestion by Haldane, (1949) that it was not a matter of coincidence, but rather a classic case of natural selection. The basic proposition of Haldane's hypothesis is that alpha+-thalassemia, is pronounced in malarious regions because it accords carriers a level of protection against death from malaria by assuaging the clinical course of the disease. What this means is that people who are not carriers or who have other forms of these conditions/disorders will most likely die, whereas carriers will survive and possibly produce more carriers, leading to a steady increase in the population of carriers in these regions.

Since Haldane's proposition, the protective effect of some of these disorders has been well documented, whereas for others, there remains lack of empirical evidence (Table 1.5).

Table 1.5. Susceptibility of Heterozygous and Homozygous alpha+-thalassaemia to severe malaria and anaemia

GENE	PROTECTION FROM SEVERE MALARIA	SEVERITY OF ANAEMIA
Heterozygous alpha+-thalassaemia. Silent carrier or mild alpha thalassaemia minor; alpha+ thalassaemia trait. (-α/αα)	Protection from severe malaria	No haematological abnormality. Hb is either normal or slightly reduced.
Homozygous alpha+ thalassaemia (-α/-α)	Protection from severe malaria	May be slightly anaemic. About 15% show slight reduction in MCV and MCH
Heterozygous α^{o} (--/αα)	No protection	Hb is either normal or slightly reduced. MCV and MCH are usually reduced.
Haemoglobin H Disease α^{o} α^{+} double heterozygous (--/ -α)	No protection	Chronic haemolytic anemia Shortened RBC life span. Hb con. is between 7g/dL and 11g/dL but may be as low as 3-4gldl.Red cells are hypochromic and show variation in size and shape MCV and MCH are reduced. Reticulocytosis is slight except during acute haemolysis. Fluctuation in Hb. Basophilic stippling always seen
Haemoglobin Bart's Hydrops fetalis or homozygous α^{o} thalassaemia (--/--)	No protection	Marked microcytic hypochromic cells, poikilocytosis, fragmented cells and numerous nucleated red blood cells

Adapted from Weatherall and Clegg, (2001)

Evidence for the protection has come principally from population studies, epidemiological and case control studies but the suggestion that alpha+-thalassaemia has been selected by malaria is mainly based on epidemiological studies (Allen *et al.,* 1997).

Min-Oo and Gros (2005) have indicated evidence from micro-epidemiological studies to suggest proportional thalassaemic prevalence with incidence of malaria amongst populations of South Pacific regions. In the view of Sakai *et al*., (2000), a micro epidemiological study conducted in Nepal indicated that the high frequency (63%) of the $-\alpha^{3.7}$ deletional alpha+-thalassaemia recorded might be due to the biological adaptation to the malaria environment.

For instance, Sickle cell trait has been documented, with a high degree of certainty, to provide up to 90% protection against death due to malaria (Hill *et al.*, 1991). Hemoglobin C was also reported to have reduced the risk of severe malaria in the Dongo people of Mali by about 80% (Agarwal *et al.*, 2000).

The same, however, cannot be said for alpha+-thalassemia, at least not with the same degree of certainty.

1.7.1 Protection of alpha^{+}- thalassaemia from severe malaria

Previous studies have expressed varied opinion on the protection from severe *Plasmodium falciparum* malaria by alpha^{+}-thalassaemia. Initially, not only was it suggested that alpha^{+}-thalassaemia offered no protection against death from malaria, it was even reported that alpha^{+}-thalassaemia predisposes children to contracting mild malaria.

Williams *et al*., (1996) indicated that both the incidence of uncomplicated *Plasmodium falciparum* malaria*, Plasmodium vivax* malaria and the prevalence of splenomegaly (an index of malaria infection) were higher in alpha^{+}-thalassaemic children compared to their normal counterparts.

In the view of Allen *et al* the incidence of contracting mild malaria among Papua New Guineans in the first two years of life was increased among alpha^{+}-thalassaemic children (Allen *et al.,* 1997). In a previous study, Allen *et al.,* (1993) suggested that heterozygous alpha^{+}-thalassaemic children from the Gambia showed no protection from either asymptomatic *Plasmodium falciparum* parasitaemia or clinical episodes of malaria.

Alpha^{+}-thalassaemia has not been found to reduce the incidence of uncomplicated malaria, does not protect from malaria infection but predisposes to mild clinical malaria. The disease has been observed to limits the progression to severe malaria anaemia (William *et al.,* 1996, 2005). In support of this observation Wambua *et al*., (2006) reported no association between alpha^{+}-thalassaemia and the incidence of uncomplicated *Plasmodium falciparum* parasitaemia or parasite densities during mild or severe anaemia episodes.

Mockenhaupt *et al*., (2001) also suggested that alpha^{+}-thalassaemia predisposed Ghanaian pregnant women to *Plasmodium malariae* infection.

They observed that the prevalence of *Plasmodium falciparum* infection and parasite densities did not show any significant correlation with alpha-thalassaemia genotypes and that *Plasmodium malariae* tended to be more frequent in alpha^{+}-thalassaemic women. Furthermore, the alpha^{+}-thalassaemia genotypes in general, did not influence *Plasmodium falciparum* malaria infection, febrile parasitaemia or parasite density (Mockenhaupt *et al*., 2004a).

Currently, there appears to be a common understanding that alpha+ -thalassaemia has an ameliorating impact on malaria. However, a seemingly contentious point is whether the observed protective effect of alpha+-thalassemia impinges upon malaria in general or only on some forms of the disease.

On this particular question, there appears to be a high degree of unanimity among the Papua New Guinean, Ghanaian, and Kenyan studies in that they all seem to suggest that the protective effect is across-the-board for all forms of malaria (Allen *et al*., 1997; Mockenhaupt *et al*., 2004a; and Williams *et al*., 2005b). Subsequent studies in Kenya clearly conflicted with the Ghanaian report but agreed with the findings from Papua New Guinea (Williams *et al*., 2005b). Another Ghanaian study, while agreeing with the protection against *Plasmodium falciparum* malaria by alpha^{+}-thalassemia, concluded that the 'impact' was specific and driven by protection from severe anaemia, the most prevalent malaria complication, whereas no effect was observed on the other forms of severe malaria (May *et al.*, 2005).

The protection conferred by alpha+thalassaemia is however, observed to be limited to the severe manifestation of *Plasmodium falciparum* malaria (Veenemans *et al*., (2008).

1.7.2 Homozygous and/or heterozygous alpha^{+}- thalassaemia protection from severe malaria

There is an apparent disagreement between researchers as to the type of alpha+-thalassaemia genotype, which provides the 'ameliorating effect'.

In Papua New Guinea where alpha+-thalassaemia genotype is almost at 90% fixation, Allen *et al*., (1997) observed protection from severe *Plasmodium falciparum* malaria with 60% and 34% risk reduction in homozygous and heterozygous children respectively.

Findings from a case control study in children living on the coast of Kenya had concluded that both heterozygous and homozygous alpha^{+}-thalassemia protect against the lethal effects of malaria, though the said protection was more evident in subjects with homozygous alpha+-thalassemia (William *et al.,* 2005). The risk for hospital death among these children was 40% lower in the heterozygous compared to 60% for the homozygous alpha^{+}-thalassaemia than in healthy children (William *et al.*, 2005).

Similar studies in Tamale, the northern part of Ghana, indicated that alpha+-thalassaemias are associated with a reduced risk for severe and fatal *Plasmodium falciparum* malaria; however, this was only true for heterozygous alpha+-thalassaemia, which occurred less frequently in severe malaria children (Mockenhaupt *et al*., 2004a). Homozygous alpha^{+}-thalassaemia did not reduce the odds of *Plasmodium falciparum* malaria. The inability to detect protection in the homozygous alpha^{+}-thalassaemia against *Plasmodium falciparum* malaria, as explained might be due to their small number and statistical limitations (Mockenhaupt *et al*., 2004a).

In a previous study, Allen *et al.,* (1993) suggested that heterozygous alpha^{+}-thalassaemic children from the Gambia showed no protection from either asymptomatic *Plasmodium falciparum* parasitaemia or clinical episodes of malaria.

A subsequent study in Tanzania also reported a reduced risk of uncomplicated malaria episodes in children homozygous and heterozygous for alpha+-thalassaemia, and the protective effect of alpha+-thalassaemia was most pronounced in children older than 5 years of age (Enevold *et al*., 2008).

1.7.3 Mechanisms of protection from severe *Plasmodium falciparum* by alpha^{+}-thalassaemia

Unlike the sickle cell trait, studies so far have not been able to provide plausible mechanisms for the protection (Pasvol, 2006, Harteveld and Higgs, 2010).

The protective mechanism of alpha+-thalassaemia however remains unclear.

Other reports have suggested various forms of mechanisms through which alpha^{+}-thalassaemia individuals confer protection against *Plasmodium falciparum* malaria.

Luzzi, *et al.,* (1991a), suggested that the protective effect of alpha+-thalassaemia is due to modified antigen recognition on the surface of the parasitized erythrocyte, with a proposal by Williams *et al.,* (1996), that the alpha+-thalassaemia may be associated with higher levels of *Plasmodium vivax* infection early in life which may have heighten immunological defences and provide better protection from more dangerous and subsequent *Plasmodium falciparum* infections.

In vitro flow-cytometry studies have indicated that erythrocytes of the alpha+-thalassaemia phenotype show reduced parasite growth and/or invasion rate (Pattanapanyasat, *et al.,* 1999) and increased binding of antibodies from malaria-immune sera (Williams, *et al.,* 2002) including enhanced splenic clearance of malaria-infected cells. Additionally, alpha+-thalassaemia RBCs age more quickly, due to oxidative stress when parasites breaks-down erythrocytes leading to membrane damage, increased phagocytosis and decreased parasite invasion (Yuthavong *et al.,* 1988; Destro-Bisol *et al.,* 1999a; 1999b; Senok *et al.,* 2006).

Furthermore, surface antigens on the RBC undergo conformational changes when the parasite enters the thalassaemic red cells and appear to bind increased amounts of immunoglobulin, which might favour early removal of parasitized red blood cells (Luzzi *et al.,* 1991a; Pattanapanyasat *et al.,* 1999; Destro-Bisol *et al*., 1999a, Destro-Bisol *et al.,* 1999b).

Severe malaria results from pathophysiological processes that include the local and systemic release of various cytokines, anaemia, decreased red cell deformability, and adhesion phenomena such as sequestration and rosetting, and the clinical features of severe malaria (such as coma, acidosis, and hypovolemic shock) (Marsh *et al.,* 1995). That alpha+-thalassaemia protects against this range of clinical manifestations of severe malaria suggests it does so through a mechanism that is central to many of these processes (Clark, *et al.,* 2003). Cockburn *et al.,* (2004) proposed that, a promoter polymorphism of red cell complement receptor 1 (CR1), an important receptor for rosetting, is significantly associated with protection from severe *falciparum* malaria.

Alpha+thalassaemia is independently associated with reduced expression of red cell CR1 suggesting that by reducing the ability of red cells to form rosettes, alpha+-thalassaemia patients, (both heterozygous and homozygous) are protected against severe malaria (Clark, *et al.,* 2003, Williams, *et al.,* 2005a).

Imrie *et al*., (2004) suggested that the higher levels of haptoglobin in the homozygous individuals were toxic to *Plasmodium falciparum in vitro*.

Further studies by Imrie *et al*., (2006) indicated that the higher level of haptoglobin (Hp), in homozygous alpha+-thalassaemic individuals confers protection by removing free haemoglobin released during haemolysis. In providing an explanation to the unusually high level of haptoglobin in homozygous alpha+-thalassaemia, Imrie and colleagues indicated that these individuals experience fewer *Plasmodium falciparum* infections than the heterozygotes thus reducing the haemolysis and increasing the haptoglobin levels.

Homozygous but not heterozygous alpha+-thalassaemia has been suggested to confer protection against the severe manifestation of *Plasmodium falciparum* parasitaemia (Fowkes *et al*., 2008). This observation was the outcome of a study conducted in Papua New Guinea, which indicated that the increased number of abnormally small erythrocytes (microcytosis) were associated with homozygous alpha+-thalassaemia that might be responsible for the protection against severe malarial anaemia (Fowkes *et al*., 2008).

1.7.4 Alpha^{+} -thalassaemia, microcytosis, and *Plasmodium falciparum* malaria

Alpha^{+}-thalassaemia is characterized by hypochromic microcytic anaemia, occurs when there is a reduction in the synthesis of the alpha-globin chains of adult haemoglobin (HbA, $\alpha_2\beta_2$) relative to β-globin synthesis; excess β-chains then form homotetramers (HbH, $\beta 4$) (Higgs *et al.,* 2001).

Microcytosis and hypochromia result from deficient haemoglobin (Hb) synthesis in erythroid cells, causing a reduction in both mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) of red blood cells.

Compared to non-thalassaemias, heterozygote alpha^{+}-thalassaemias do not present any clinical symptoms, whereas homozygote alpha^{+}-thalassaemias are characterized by mild hypochromic microcytic anaemia (Imrie, 2006).

The microcytic hypochromic anaemia observed in alpha^{+}-thalassaemia could be due to iron deficiency resulting from an increased rate of haemolysis leading to a fall in plasma haptoglobin levels and the excretion of free haemoglobin in urine thereby reducing the body's iron stores (Imrie *et al*., 2006).

Both iron deficiency and alpha^{+}-thalassaemia can therefore cause microcytosis (Colah *et al*., 2010) however, iron deficiency remains the first cause of microcytosis whilst deletional αlpha^{+}-thalassemia, the most frequent haemoglobinopathy throughout the world, represents the second most frequent cause of microcytosis followed by β-thalassemia heterozygous state. Without an increase in Hb A_2 levels, these haematological alterations may be due to α-thalassaemia or iron deficiency (Borges, *et al.,* 2001).

Ronald (2006) had suggested a careful three tier approach involving: (1) Full blood count (2) Special haematological tests and biochemical iron markers, followed by (3) DNA mutation analysis, that may provide the most effective way in which to detect primary gene mutations. For those with red cell microcytosis, a reliable measure of iron status is required (e.g. serum ferritin) to exclude iron deficiency (Vulliamy 2001). The red cell indices are suggested to be important indicators in the assessment of thalassaemia trait, which is characterized by microcytosis (Howard, 2005; Modell and Darlison, 2008). This is because microcytosis may be due to iron deficiency but also heterozygous or homozygous alpha^{+} -thalassaemia or alpha^{0}-thalassaemia trait (Wonke *et al.,* 2007, Eivazi-Ziaei *et al*., 2008). Low MCV (mean corpuscular volume) or MCH (mean corpuscular haemoglobin) might provide a clue for thalassaemia especially in regions at risk, and iron deficiency must be excluded as a first step (Bergstrome and Poon, 2002).

Though the subject of alpha+-thalassaemia and anaemia in Africa is still to be fully understood, a study carried out in southwest Nigeria reported that alpha^{+}-thalassemia contributes essentially to mild anaemia, microcytosis and hypochromasia (Mockenhaupt *et al*., 1999a). In another study, Rahim, (2008), found a considerable (20%) prevalence of alpha+-thalassaemia in microcytic hypochromic anaemic patients.

Luzzi *et al*., (1990) indicated an unrestricted growth of *Plasmodium falciparum* in microcytic erythrocytes in alpha thalassaemia.

They explained that whilst morphological abnormalities may be detected in parasitized thalassaemic erythrocytes, development of *Plasmodium falciparum* in the condition is not limited by microcytic red blood cells.

Later, Mockenhaupt *et al*., (1999b) observed that microcytosis was significantly associated with protection from haemoglobin decrease due to *Plasmodium falciparum* and that the rate of infection was lower in microcytic than in normocytic anaemia. Microcytosis has therefore been suggested to be responsible for the protection against severe malarial anaemia (Mockenhaupt *et al*., 1999b).

Fowkes *et al*., (2008) corroborated this finding by indicating that the protection against severe malarial anaemia in homozygous alpha+-thalassaemia was driven by red cell microcytosis.

1.7.5 Iron deficiency and malaria in children

Both iron deficiency and malaria are common in much of sub-Saharan Africa, and the interaction between these conditions is complex (Nyakeriga *et al*., 2004b).

Iron-deficiency anaemia is also a major threat to maternal and child health. Overall, 78 percent of Ghanaian children ages 6-59 months have some level of anaemia, including 23% of children who are mildly anaemic, 48% who are moderately anaemic, and 7% who are severely anaemic (Ghana Demographic Health Survey, 2008).

Earlier reports by Hendrickse *et al*., (1989) on the effects on malaria of treating iron-deficiency anaemia with oral iron in Gambian children suggested that there was a significantly increased risk of fever associated with severe malarial parasitaemia for children with iron-deficiency anaemia when given iron. Iron chelators were suggested to inhibit malarial growth *in vitro* as well as *in vivo* (Hershko *et al.*, 1988), and later reported cases of an *in vitro* study indicated that a small labile pool of iron in red cells (crucially smaller in iron deficiency) provided the iron the malaria parasite requires (Loyevsky *et al.,* 1999)**.**

Cheesbrough (1999) then suggested iron as essential for the growth of *Plasmodium falciparum* and that higher levels in serum following treatment with iron were shown to increase the incidence of malaria

In contrast, Tsedal *et al*., (2004) observed that low dose daily iron supplementation did not increase the prevalence of malaria infection or density of parasites in young Zanzibari children.

However, recent observations from a study in Zanzibar, Tanzania (Sazawal *et al*., 2006) indicated that oral iron-folic acid supplements might increase the risk of morbidity from malaria and/or the bacterial infections that complicate malaria in children below 3 years of age.

The administration of iron parenterally or orally without food may increase plasma iron concentrations and transferrin saturation resulting in plasma iron exceeding the binding capacity of transferrin leading to the appearance of non transferrin-bound iron (NTBI).

This is potentially toxic in promoting free radical formation and becoming readily available to pathogens (WHO, 2006). A further explanation by WHO is that iron deficiency may impair the immune response while an iron bolus increases the risk of forming potentially toxic NTBI.

Iron-deficient children may then be protected from toxic effect of NTBI because iron is removed more rapidly from the plasma due to their higher tissue requirements for iron.

Increase exposure to phosphatidylserine in individuals who were iron deficient and infected with *Plasmodium falciparum* have resulted in red cell death in humans (Koka *et al*., 2007). With the reported *in vitro* impairment of intraerythrocytic growth and infection of erythrocytes, Koka *et al*., (2007), using mice models reported that iron deficiency influences the course of malaria in *Plasmodium falciparum* berghei infected mice by a decrease in parasitaemia and accelerated clearance of parasitized red cells. However in a cohort of children in coastal Kenya Nyakeriga *et al*., (2004b) had earlier reported that iron deficiency was associated with protection from mild clinical malaria. This they explained might be due to a complex interaction exists between the parasite, iron status and immune system of the individual.

The immune response to malaria, as suggested by Sazawal *et al*., (2005), does not solely depend on access of the parasite to free plasma iron in the intraerythrocytic phase of infection.

In malaria-endemic areas, there is also some evidence that an elevated plasma zinc protoporphyrin/haem (ZPP/H) ratio may identify iron-deficient children (Labbe, 1992) who will benefit from iron supplementation.

This is due to the fact that there is incorporation of zinc into protoporphyrin instead of iron in iron deficiency.

Iron deficiency, which results in decreased production of red blood cells, can therefore be said to offer some protection to the host against malaria (Rodak 2002, Cromer *et al*., 2009)

1.8 Aims of the research

The aim of this study was to test the hypothesis that the protective effect of alpha+-thalassaemia against *Plasmodium falciparum* parasitaemia is driven by microcytosis and hypochromasia that might result in reduced parasites density.

1.8.1 Specific aim 1

This study would attempt to establish whether the heterozygous, homozygous or both alpha+ globin genotypes confer protection from severe malarial parasitaemia

1.8.2 Hypothesis 1

That both homozygous and heterozygous alpha+-thalassaemia genotypes are protected from *Plasmodium falciparum* parasitaemia.

1.8.3 Specific aim 2

To determine the effects of microcytosis and hypochromasia in protecting alpha+-thalassaemia and non thalassaemia patients from severe *Plasmodium falciparum* malaria and the relationship between MCV and geometric mean parasite density (GMPD)

1.8.4 Hypothesis 2

That microcytosis and hypochromasia are responsible for protection from severe malaria amongst alpha+ thalassaemia and non-alpha thalassaemias

1.9 Objectives

- To estimate G6PD deficiency, HbC and HbAS frequency in subjects, in order to exclude them from the analysis, as these confounding factors protect against *Plasmodium falciparum* malaria
- To estimate the mean parasite density in subjects and compare with microcytic and normocytic, and hypochromic and normochromic children
- Characterise the genotype specific protection from severe *Plasmodium falciparum* malaria
- Determine whether the protective effect of alpha+thalassaemia from *Plasmodium falciparum* wane with age

2.0 Materials, subjects, and methods

2.1 Location of Project and Subject Population

Subjects were patients already attending the polyclinic out patient department at Komfo Anokye Teaching Hospital (KATH) as well as the children hospital at Manhyia where their blood samples were already being collected for medical care. Samples were analyzed at the KATH and the Kumasi Center for Collaborative Research in Tropical Medicine (KCCR). Both institutions are located in Kumasi – the second largest and fastest growing city in Ghana, with a projected population of about 645,100 (Ghana Statistical Service, 2010). KATH is a major tertiary referral Hospital in Ashanti region, while KCCR is a major research facility within the College of Health Science of the Kwame Nkrumah University of Science and Technology. In Kumasi ethnic Akans, make up 77.7% of the population, while other smaller groups, including, but not limited to, the Mole-Dagbon (9.1%), Ewe (2.9%), and Grusi (2.9%), account for the rest. One thousand six hundred and seventy-two (1672) children, aged ≤10 years, were recruited as subjects for this study. Seven hundred and thirty-two (732) cases that tested positive to *Plasmodium falciparum* malaria were enrolled.

2.2 Study Management

The Investigator, Clement Opoku-Okrah, managed the study locally with Prof. Tsiri Agbenyega the local supervisor who constituted the Local Steering Committee that oversaw the day-to-day conduct of the study, whilst Dr. Caroline Smith, Dr., Mike Gordge, and Dr. Martin Parry constituted the external supervisors. Dr. Caroline Smith however visited Ghana to monitor the progress of the project. The Local Steering Committee met monthly

2.3 Ethical Consent

Ethical clearance was obtained, from the Committee on Human Research Publication and Ethics, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana (Approval letter: CHRPE/KNUST/KATH/12_03_09), Approval was given by the University of Westminster-Ethical Committee (Application number- 08/09/14) for all experimental works (refer to appendix 5). Subjects were patients already attending the polyclinic out patient department of the Komfo Anokye Teaching Hospital (KATH) as well as the children hospital at Manhyia, all in Kumasi, Ghana, at where their blood samples were already being collected for medical care. Blood samples were collected from the subjects only after an informed consent was given by their parents/guardians.

2.4 Informed consent

Parents were duly informed about the research and their involvement in the study was purely voluntary. Informed consent were sought from mothers before samples were taken.

2.5 Inclusion Criteria

Children with proven malaria within the specified age group, and whose parents had consented, were included in the study. Subjects were not more than 10 years of age at enrolment. The problem of malaria infection is most critical for children less than five years of age.

2.6 Exclusion Criteria

Children with other serious chronic disease were excluded because their illness may confound the results obtained for this study. Those whose parents did not consent, or those older than 10 years old, were not included either. In addition, children that were known to have HIV/AIDS and whose clinical conditions (HIV/AIDS and Hepatitis B) were indicated on their request form were exempted before sampling, although their HIV/AIDS could not possibly have influenced tests.

2.7 Risk

There is no inherent major risk to the subjects. Blood sampling by venepuncture for tests may cause bruising, infection, fainting, pain, or discomfort.

The pain of blood sampling by venepuncture is minimal but is real and unfortunately an unavoidable part of health care.

All normal precautions were taken to keep these side effects from happening. 4mls of blood was taken from each child.

2.8 Sample collection and experimental approach

Haematological and parasitological tests were performed within 24 hours of collecting blood sample for malaria parasite density, complete blood count, Glucose-6-phosphate dehydrogenase (G6PD) screening and Hb electrophoresis. The rest of the sample was stored at -20 ^{o}C for the determination of alpha globin genotype.

Samples were collected between May 2009 and July 2011. The nature of the study was explained to the parents/guardians of the sequential patients and their child/ward's participation enlisted. With informed consent, 4 ml of blood was collected into ethylenediamine tetra-acetic acid (EDTA) through venipuncture.

Three milliliters of blood was put into the one tube for the purpose of the malaria parasite test, complete blood count (CBC), and the ferritin tests, while the remaining 1 ml was placed into a second tube to be used exclusively for the polymerase chain reaction (PCR). After the blood was collected, details like the age, sex, and location were also recorded to help with the analysis of the data. Blood samples were placed in an ice-chest and taken to the main haematology laboratory at KATH for immediate testing. The tests done at KATH include malaria parasite test, CBC, and the ferritin test.

The samples that were set-aside for the PCR were then transported to the Kumasi Center for Collaborative Research in tropical Medicine, where DNA was extracted and genotypes determined by Single-Tube Multiplex PCR method (Chong *et al*., 2000)

2.81. Parasitological studies

2.8.1.1 Experimental approach by Giemsa staining technique for *Plasmodium falciparum* parasitaemia

The asexual form of *Plasmodium falciparum*, which is the predominant Plasmodium species, found in Ghana was tested. The malaria parasite test was carried out within 45 minutes of sample collection. Thick and thin blood films were prepared for all the 1672 samples, using clean and grease-free slides and labelled in accordance with the recommendations of Agomo *et al.,* (2001).

Both films were stained with 10% Giemsa (pH 7.2) and read for malaria parasites following standard, quality-controlled procedures (WHO, 2009) where thin and thick blood films of each sample were applied on a single pre-cleaned slide. Fixation of thin blood films was done with methanol and thin and thick blood films were stained with 10% Giemsa at pH 7.2 .The stained slides were examined using a light microscope with x100 oil immersion objective for *Plasmodium falciparum* trophozoites (Figure 2.1)

The results were initially recorded as either positive (+ve) or negative (-ve). Malaria parasites density was determined on all positive cases.

Using thick film**,** 200 WBC were counted and the number (N), of parasite seen noted. Two hundred (200) WBC then divides the N and then the value multiplied by the WBC count of the subject or WHO standard for WBC, which is 8000/µL.

In this study the RBC count or the total WBC count of the subjects was used to calculate the parasite density. The WHO standard for WBC was not used as it is meant for facilities that did not have equipment for estimating the total WBC count.

Using the thin film, 1000 RBC's were counted and the number of parasitized RBC's counted along side simultaneously. The total number of parasitized red cells is then divided by 1000 and the value multiplied by the RBC count of the subject to get the parasite density.

Parasitaemia was expressed as the number of asexual forms of the malaria parasites per microliter. It was graded as low (1-999/µL), moderate (1000-9999/µL), high (10000-99999/µL) and severe (≥ 10000/µL).

An independent parasitologist at the Malaria Research Laboratory at KATH further read malaria slides that were initially negative for malaria parasites. Twelve of the slides with no malaria parasites seen were however reported as positive by the independent parasitologist. No positive slides were sent for independent confirmation, as these were very clear on both the thin or thick film.

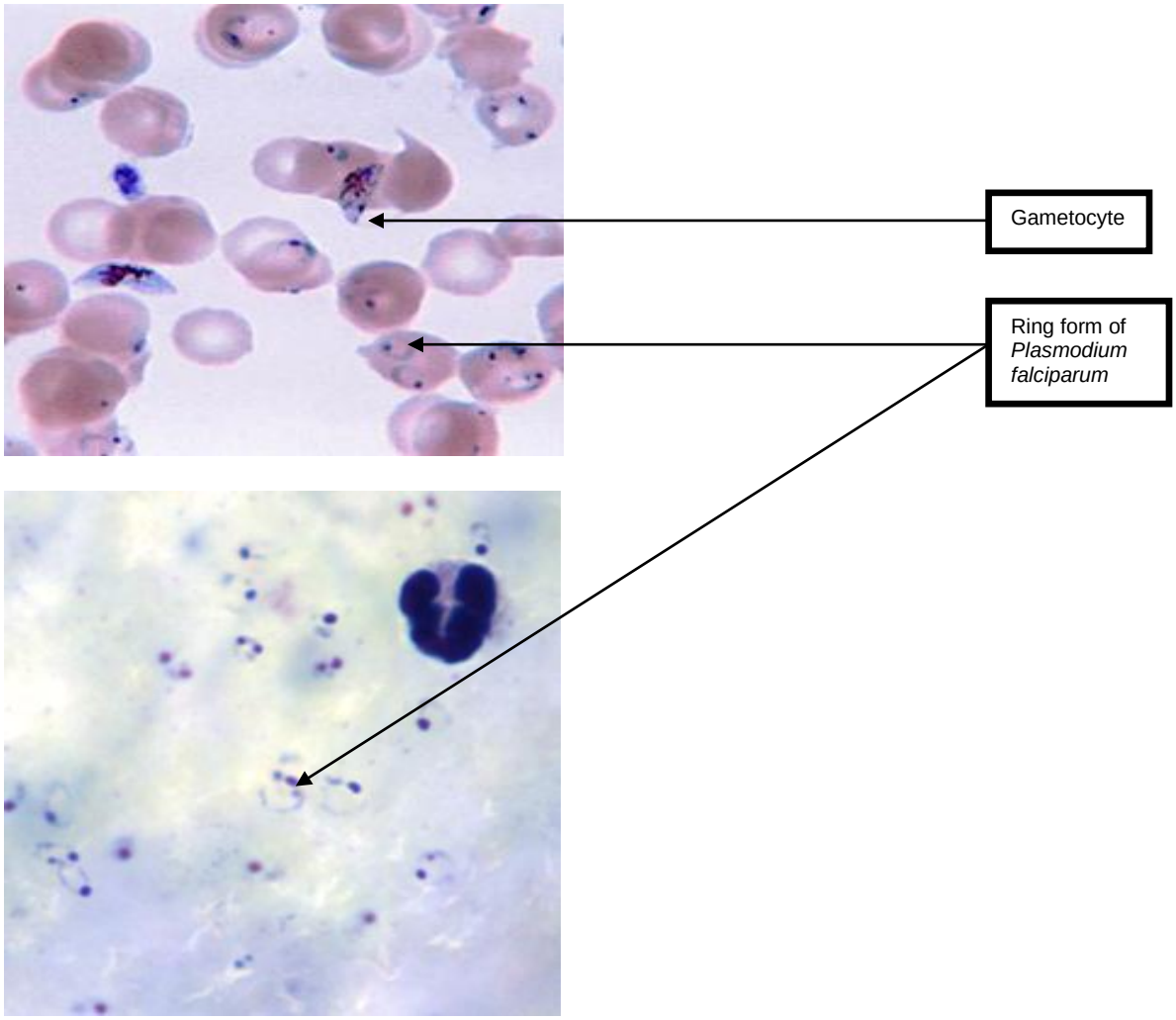

Figure 2.1 Trophozoite of *Plasmodium falciparum* in affected red blood cell in A-thin film and B-thick film x100

2.8.2 Haematological studies

2.8.2.1 Experimental approach for glucose-6-phospate dehydrogenase (G6PD) screening by methaemoglobin reductase method

The oxidation of haemoglobin to methaemoglobin by sodium nitrite and the subsequent enzymatic reconversion to haemoglobin in the presence of methylene blue was determined by the Methaemoglobin Reductase Method for G6PD screening (Brewer *et al*., 1962; Bhasin and Chahal 1996)**.**

The test was set up within 30 minutes after blood sample has been taken. Samples that could not be worked on were kept at 4^{O}C but were tested within 6 hours. Two millilitres of blood sample was placed in a test tube and 0.1 ml of 0.18 M sodium nitrite solution added followed by 0.1 ml of 0.0004 M methylene blue chloride solution.

The test was controlled for full defect, partial defect and no defect using known samples. Both test and control were incubated at 37^{o}C for 3 hours. Aeration was ensured at 60, 120 and 180 minutes during incubation.

After incubation, 0.1 ml each of the test sample and the controls were pipetted into 10 ml distilled water in separate clean glass test tubes of identical diameter. The contents were mixed gently and the colours observed and compared (Figure 2.2)

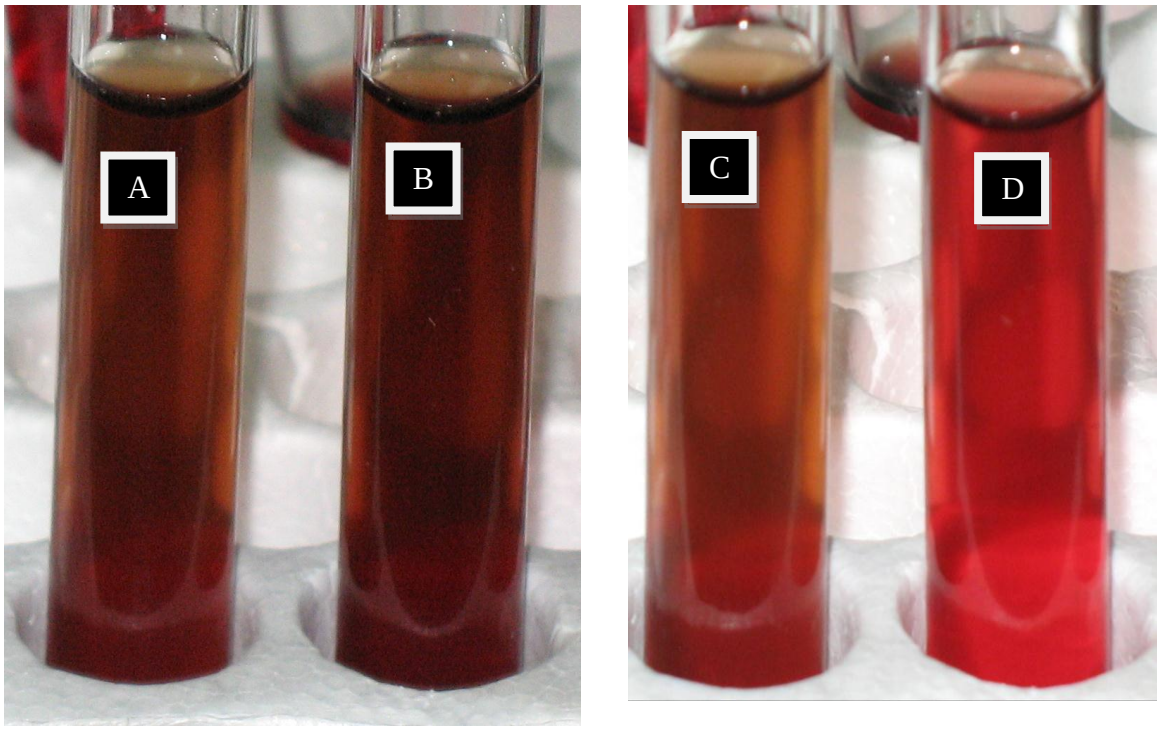

Figure 2.2 Glucose-6-phosphate dehydrogenase (G6PD) deficient testing by methaemoglobin reductase method**.**
A- Positive control**; B-**full defect**; C-**partial defect and **D-** no defect

No defect or normal sample was clear red

Heterozygous female was light dark grey or light brown.

Hemizygous males or homozygous female was dark grey or brown.

2.8.2.2 Experimental approach for the determination of β-globin genotypes by alkaline cellulose acetate Hb electrophoresis

Haemoglobin AS (HbAS) and haemoglobin AC (HbAC) trait individuals are protected from the effect of severe malaria. They are serious confounders in epidemiological studies of αlpha+-thalassaemia in Africa, and needed to be excluded (William *et al*., 2005c, Wambua *et al*., 2006). Beta thalassaemia was excluded because facility for identifying the disease was not available at the time of this study.

The phenotypes were determined using the cellulose acetate method (Kohn *et al.,* 1969) by alkaline haemoglobin electrophoresis (Helena, Sunderland, and Tyne & Wear). The Titan® Cellulose Acetate membrane (76 mm x 60 mm) was soaked in distilled water, blotted then soaked in the Tris-EDTA-borate. Haemolysate was prepared by adding 0.005M EDTA in deionized water with 0.07% potassium cyanide to whole blood, and was applied to the Titan III® Cellulose Acetate Plate.

The haemoglobin electrophoresis was performed at 350 volts for 25 minutes using an alkaline buffer (pH 8.2-8.6) and were stained with (0.5% (w/v) Ponceau S in an aqueous solution of 10% (w/v) sulfosalicylic acid and the migration of the haemoglobin noted. Control sample containing haemoglobins A, F, S and C were included with each electrophoresis run (Figure 2.3)

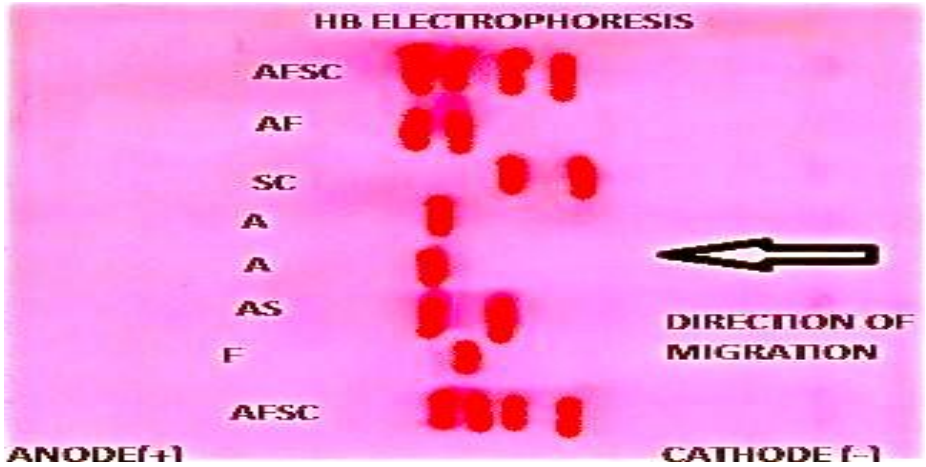

Figure 2.3: Haemoglobin electrophoresis by cellulose acetate method at p^H 8.6 showing haemoglobin A, haemoglobin AS (sickle cell trait), haemoglobin S, haemoglobin C and haemoglobin F. Haemoglobins AFSC were pooled together and used as control. The arrow shows the migration of negatively charged haemoglobins from the cathode to the positively charged anode**.**

2.8.2.3 Experimental approach for complete blood count (CBC)

To determine the level of anaemia, haemoglobin (Hb), mean cell volume (MCV), mean cell haemoglobin (MCH), and red cell count (RBC) counts were estimated using Cell-Dyn® 3500 (Abbott Diagnostics, USA). Samples with Hb level ≤5.0g/dL were considered as severe anaemia cases.

Microcytosis is defined in this study as MCV ≤ 76 fL and hypochromasia as MCH ≤ 25 pg were used to exclude microcytosis and hypochromasia. These values are established cut-off values in use for children at the Komfo Anokye Teaching Hospital of Kumasi, Ghana.

2.8.3 Alpha globin genotyping

2.8.3.1 Experimental approach for alpha globin genotyping by polymerase chain reaction (PCR)

The characterization of the -α $^{3.7}$ kb deletional α+- thalassaemia (heterozygous, homozygous and wild type) were determined by DNA analysis of each deletion breakpoint using the single-tube -PCR assay that allowed a high throughput genetic screening for these common haematological disorders (Chong *et al*., 2000. Genomic DNA was extracted strictly in compliance with the protocol described in the QIAamp DNA Blood Mini kit Handbook (2003). Sample was incubated at room temperature and 20 µL of Proteinase K (a lysis enzyme) added, followed by the addition of 200 µL of blood sample and 200 µL of Lysing buffer (buffer AL) then, 200 µL of ethanol (96-100%). 500 µL Residual washing buffer 1 (Buffer AW1) was added followed by 500 µL Residual washing buffer 2 (Buffer AW2) and finally 200 µL Elution buffer (Buffer AE) added, and an elute of genomic DNA collected and stored at -20 ^{O}C.

Following the extraction of the DNA, each DNA sample was amplified using a Thermal Cycler (Eppendorf, Germany). Along with the test sample, each PCR run included three positive control DNAs, Homozygous (mutant) DNA (64 ng/µL), normal genotype (wild type) DNA (71 ng/µL), and heterozygote DNA (105 ng/µL)) and a negative control of aqua ad injectabilia. The total reaction volume was 50 µL in adherence to the protocol of Chong *et al*., (2000).

The primers used in the PCR to amplify the 1800bp and the 2000bp for the heterozygous, homozygous and normal genotypes are the α^2/3.7-F 5' CCCCTCGCCAAGTCCACCC 3', 3.7-R 5' CCCCTCGCCAAGTCCACCC 3' and α2-R 5' AGACCAGGAAGGGCCGGTG 3'

Each 50-µl-reaction master mix tube contained 5 µL 10 x PCR buffer (QIAGEN), 10 µL of Q-solution 5 x (QIAGEN), 1 µL of 1 forward primer α^2/3.7-F (10 pmol/µL), 2 µL of 1 reverse primer $\alpha^{3.7}$-R (10 pmol/µL) (MWG-Biotech AG),1 µL of 2 reverse primer α^2-R (10 pmol/µL) (MWG-Biotech AG), 1 µL of dNTPs (10 mM each), 0.25 µL of Taq polymerase (5 U/µL) (QIAGEN), 1 µL of DNA template, and 28.75 µL of aqua ad injectabilia. The amplification conditions were as follows: 5 minutes of initial denaturation at 95 ^{o}C, followed by 40 cycles of 45 seconds denaturation at 95 ^{o}C, 1 minute 15 seconds annealing at 57 ^{o}C and 2 minutes 30 seconds of extension at 72 ^{o}C, and a 10 minutes final extension at 72 ^{o}C. It was then held at 8 ^{o}C. Following amplification 10 µL of each amplicon and 10 µL of the 100 bp ladder were electrophoresed through 1.5% agarose gel 1x TBE gel at 80 volts for about 45 minutes, stained in ethidium bromide, visualized using an ultraviolet transilluminator (KW 245) attached to a monitor. The images were then printed out on a black and white Polaroid film (Figure 2.4). The individual bands were then compared with the positive controls, and the different genotypes determined. The extent of deletion was confirmed by comparing the bands with the 100 base pair (bp) DNA ladder.

Along the 100 bp DNA ladder, the 1800 bp point was the normal genotype (wild type), the 2000 bp was the homozygous (mutant) type, while the heterozygote form was comprised of two strands, one of the 1800 bp, while the other was of the 2000 bp. The results were thus reported as Normal genotype (αα/αα), Homozygous (-α/-α), and Heterozygote (-α/αα).

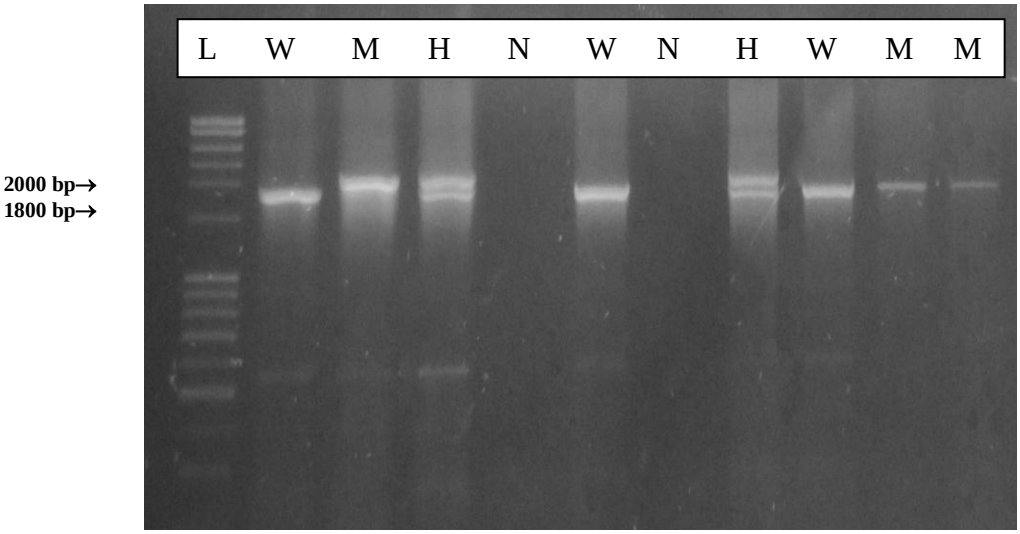

Figure 2.4: Ethidium bromide-stained gels showing results from multiplex PCR for the $\alpha^{3.7}$ thalassaemia. Sizes of the amplified fragments are given in base pair (bp). Lane L is the 100 bp DNA; M- Mutant (2000 bp); H – Heterozygote (1800 bp and 2000 bp); W- Wildtype or normal genotype (1800 bp); N- Negative

2.9 Statistical analysis

All data were entered in Microsoft excel and were analysed using SPSS 17 (IBM Corporation, New York, USA). Statistical analyses for variables that were not normally distributed were handled by the Shapiro Wilks W test to identify those with skewness after which they were log transformed to better meet the assumptions of hypothesis testing. The prevalence and frequency distribution of the haemoglobin phenotypes, G6PD deficiency and alpha+ thalassaemia for gender and malaria parasitaemia were summarized by descriptive analysis.

Geometric Mean Parasite Density (GMPD) was determined after the log transformation of the mean malaria parasite density.

The differences between haemoglobin, RBC, MCV, MCH and GMPD by normocytic and microcytic, hypochromic and normochromic and the alpha+ thalassaemia genotype children were determined by one-way ANOVA. Post-hoc test were performed using the Tukey for multiple comparisons. The strength in the relationship between GMPD and MCV, MCH, RBC and haemoglobin levels was determined by Pearson correlation coefficient. Significance was estimated by 95% confidence interval ($p< 0.05$)

Chapter 3

3.0 Baseline characteristics of study population

3.1 Introduction

Some red blood group polymorphisms are known to confer resistance against severe malaria infection. However, the theory of balance selection for the sickle cell trait, Glucose-6-phosphate dehydrogenase (G6PD), haemoglobin AC (HbAC), and other blood group genotypes appear to be substantiated, there is still the need to determine the prevalence of these RBC polymorphisms to *Plasmodium falciparum* malaria.

G6PD deficiency is the most common human enzymeopathy in the world (Ruwende and Hill, 1998) and mutations in the gene are varied between populations with epidemiological studies supporting associations between malaria resistance and deficiency of the enzyme (Mehta *et al.,* 2000; Aidoo *et al*., 2002).

The Africa type (G6PD A-) has however been indicated to confer protection against malaria in male but not female children who have just one abnormal G6PD gene, and against cerebral malaria (Guindo *et al.,* 2007) as well as providing protection in pregnancy of heterozygous deficient women (Mockenhaupt *et al*., 2003). Some mechanisms have been suggested to drive this protection, which includes inhibition of parasite multiplication by oxidized toxic substances in infected red blood cells that make them susceptible to phagocytosis or haemolysis (Cappadoro, *et al.*, 1998; Miller *et al*., 1984; Golenser *et al.,* 1988).

The susceptibility of the G6PD patient red blood cells to phagocytosis by peripheral blood monocytes and the increased binding of IgG and complement C3 to infected red cells have been suggested to confer protection against severe malaria (Schwarzer *et al*., 1992). A few studies in Ghana have reported various rates of prevalence of G6PD deficiency (Allison *et al.,* 1961, Lewis *et al.,* 1965, Acquaye *et al.,* 1973, Burchard *et al.,* 2001, Baird *et al.,* 2002). Current studies have suggested prevalence rates of 8.33% in the northern part of Ghana (Baird *et al.,* 2002), 19.0% in the middle belt of Ghana (Amoako *et al.,* 2010, personal communication) and 30.4% and 2.6% heterozygous and homozygous G6PD deficient pregnant women in the Ashanti region of Ghana respectively (Mockenhaupt *et al*., 2003).

Africa's burden of sickle cell disease is about 200,000 out of a projected annual world population of about 300,000 (WHO 2006). About 70% of the sickle cell anaemia (SCA) occurs in sub- Saharan Africa where recent reports suggest that 50% to 80% of affected children die annually (Makani *et al*., 2009).

Current estimates suggest that the frequency of the sickle-cell trait (HbAS) in Ghana and Nigeria is 15% and 30% respectively (WHO 2006).

In Ghana, the newborn screening for sickle-cell disease, which started in February 1995 in the Ashanti region of Ghana, had by December 2011 screened 345,861 infants. Prevalence for sickle cell disease (SCD) was 1.9%, haemoglobin FS (HbFS), 1.0%, haemoglobin FSC (HbFSC), 0.8% and 0.008% for haemoglobin SA (HbFSA). Prevalence for haemoglobin FAS (HbFAS) was 13.3%, haemoglobin FA (HbFA), 75.6% and haemoglobin FAC (HbFAC), 8.9% (Ohene-Frempong 2012, Unpublished data).

Mockenhaupt *et al*., (2000) observed a 14% prevalence of HbAS, 7% of HbAC and 1% of HbSS in pregnant Ghanaian women. Amoako *et al*., (2010) (personal communication) had reported a 9.2% prevalence of HbAS amongst children in the Kintampo North Municipality of Ghana. A recent study involving a cohort of children in the Ashanti region of Ghana however, reported a 10.9% prevalence of HbAS (Kreuels *et al*., 2010). Sickle cell disease has been described as a double–edged sword with evidence suggesting that those with the sickle cell trait (HbAS) are protected against malaria whilst those with HbSS not only suffer severe health problems but also generally die from malaria (McAuley *et al*., 2010). HbAS individuals are not only protected against the severe effects of malaria due to an early and enhanced acquisition of protective immunity (Aluoch 1997), they also enjoy significant protection against severe malarial anaemia, and high-density parasitaemia (Aidoo *et al.,* 2002). Contrary to other observations the homozygous sickle-cell state (HbSS) and the sickle cell trait (HbAS) were significantly associated with reduced risks of high-density parasitaemia (>10,000 parasite/µL) Aidoo *et al.,* (2002) and lower parasite rate than compared to Hb AA.

This observation by Aidoo and his co-workers did not support earlier suggestions by Mockenhaupt *et al*., (2000) who reported a higher malaria parasite density in pregnant Ghanaian women with HbAS than those with Hb AA.

HbC is commonly found in the hyperendemic malaria region of West Africa and the mutation presents with mild haematological symptoms in the homozygous state (Robert *et al*., 2004).

Very few studies have reported the prevalence of HbAC in Ghana. Kreuels *et al*., (2010) reported a 10.5% prevalence of HbAC in a cohort of children in the Ashanti region of Ghana.

HbC has been suggested to reduce the risk of developing severe *Plasmodium falciparum* by 80% in the Dogon people of Mali (Agarwal, *et al*., 2000) and 29% and 93% reduction among Burkinabe population who were HbAC and HbCC respectively (Modiano *et al*., 2001). In Ghana, a risk reduction of 47% had been reported (Mockenhaupt *et al*., 2004 a) among the people of Tamale. These findings therefore suggest a protective effect against *Plasmodium falciparum* for heterozygous and homozygous HbC in certain populations of West Africa.

However, among the people of Agogo in the Ashanti region of Ghana, (Kreuels *et al.,* 2010) did not observed protective effect against uncomplicated *Plasmodium falciparum* malaria in HbC children suggesting that population-specific factors, including differences in genetic background, may have a strong influence on the distributions of haemoglobinopathies in malarious regions.

The objectives of this study were:

- To estimate in a cohort of children from Kumasi metropolitan area
    - The prevalence of erythrocyte polymorphisms
    - The prevalence of G6PD
    - The prevalence of alpha+ thalassaemia
    - The prevalence of *Plasmodium falciparum* parasitaemia

3.2 Subject populations and experimental approach

Participant recruitment and blood sample collection followed the procedure described in section 2.1 of chapter 2

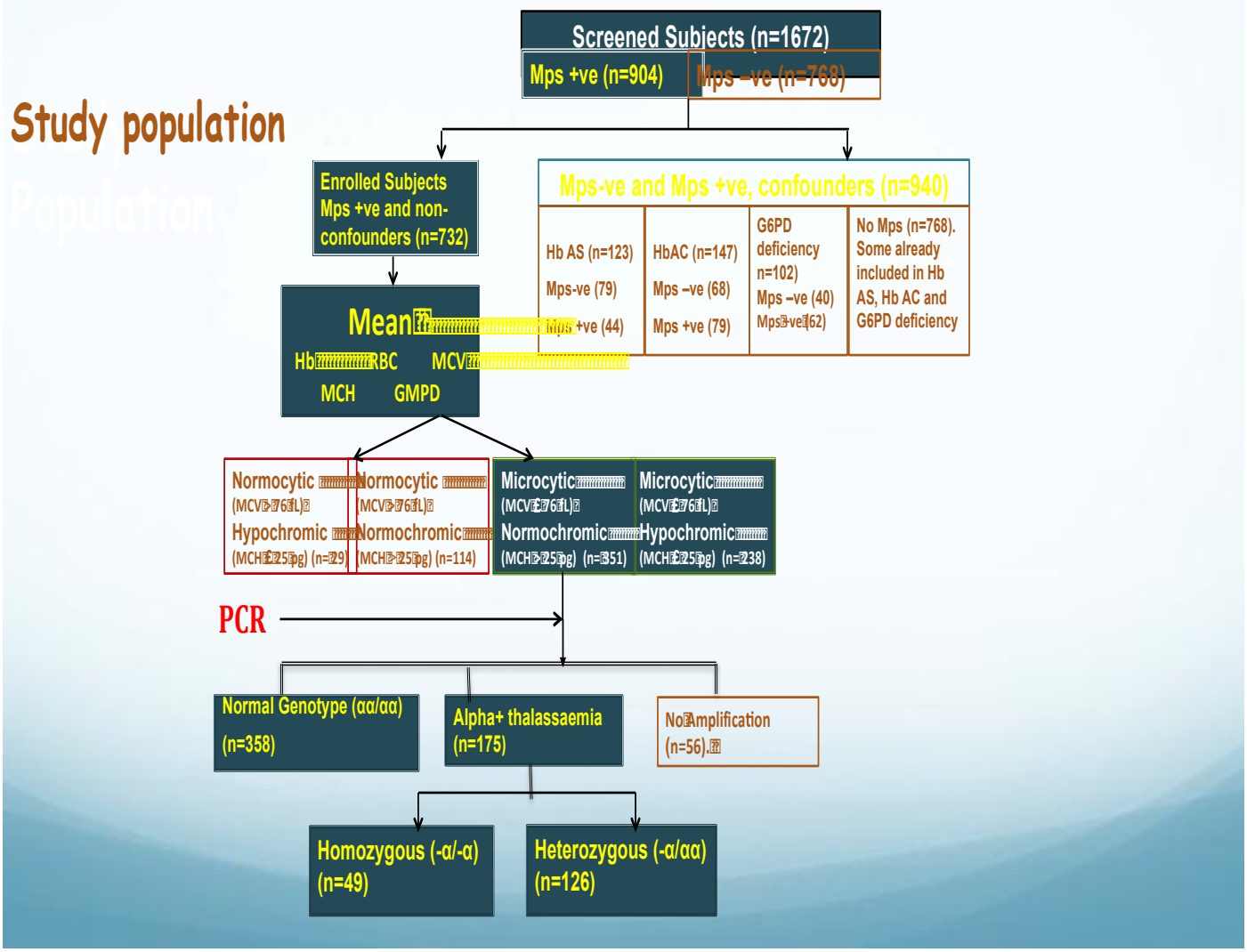

Figure 3.1: Flow diagram of study population, eligibility, confounders and tests used. Hb-haemoglobin, RBC- red blood cell, MCV- mean cell volume, MCH-Mean haemoglobin concentration, HbAS-haemoglobin AS, HbAC- haemoglobin AC, GMPD-geometric mean parasite density; α/αα, heterozygous alpha +- thalassaemia; -α/-α, homozygous alpha+-thalassaemia; αα/αα, normal or wild type alpha +- thalassaemia. n is number of subjects. Mps refer to malaria parasite (+ve is positive and –ve is negative). PCR is polymerase chain reaction. **Only children who were microcytic were tested for alpha thalassaemia**

3.3 Experimental protocol

3.3.1 Assessment of *Plasmodium falciparum* malaria

Malaria parasite density was determined using the thick and thin blood films by the microscopic method. This followed the experimental procedure as describe in section 2.8.1.1

3.3.2 Evaluation of G6PD activity by the Methaemoglobin Reductase Method

G6PD deficient individuals are also protected from the effect of severe malaria, and could be a serious confounder and therefore need to be excluded. Tests were performed within 30 minutes after sample collection as indicated in the experimental procedures in section 2.8.2.1

3.3.3 Determination of β-globin phenotypes by Alkaline Cellulose Acetate Hb Electrophoresis

The phenotypes were determined using the cellulose acetate method at pH 8.6 (Kohn *et al.,* 1969) by alkaline haemoglobin electrophoresis (Helena, Sunderland, and Tyne & Wear)**.** The subjects' red cell haemolysate were prepared and haemoglobin electrophoresis done as indicated in the experimental procedure in section 2.8.2.2.

3.3.4 Polymerase chain reaction (PCR)

Genomic DNA was extracted strictly in compliance with the protocol described in the QIAamp DNA Blood Mini kit Handbook (2003). The characterization of the $\alpha^{3.7}$ kb α+-thalassaemia (heterozygous, homozygous and wild type) was determined by DNA analysis (Chong *et al*., 2000) as explained in section 2.8.3.1 of the experimental protocol.

3.4 Statistical analysis

All data were entered in Microsoft excel and were analysed using SPSS 17 (IBM Corporation, New York, USA)..

The prevalence and frequency distribution of the haemoglobin phenotypes, G6PD deficiency and alpha+ thalassaemia for gender and malaria parasitaemia were summarized by descriptive analysis. All confounders based on haemoglobin genotypes, G6PD deficiency and malaria parasite negative were analysed and excluded from further analysis.

3.5 RESULTS

One thousand six hundred and seventy-two (1672) children of mean age 51.2 months (range, 1-120 months) were screened. Initial screening of subjects was performed to exclude G6PD deficiency (Table 3.1), Hb AS and Hb AC (Table 3.2) individuals who are known to be protected from the effect of severe malaria and are serious confounders in epidemiological studies of alpha+-thalassaemia in Africa and therefore were not enrolled into the study. *Plasmodium falciparum* parasitaemia was not seen in 768 (45.9%) of the children tested, whilst 904 (54.I %) of the children tested positive for *Plasmodium falciparum* parasitaemia out of which 732 were enrolled. These excluded confounders who were also positive for parasitaemia. All malaria positive children were tested for haemoglobin level; RBC count, MCV, MCH and geometric mean parasite density (GMPD) was determined.

MCV (cut-off ≤ 76 fL) was used to discriminate microcytic (589/732) from normocytic (143/732) subjects and MCH (cut-off ≤ 25 pg) used to distinguish hypochromic (267/732) from normochromic (465/732) children (Figure 3.1).

All microcytic children were subjected to α-globin genotyping by PCR of which 358 were normal genotypes, 49 homozygous alpha+-thalassaemia and 126 heterozygous alpha+-thalassaemia. Fifty-six of the subjects did not show any amplification for PCR (Figure 3.1) and were therefore excluded from further analysis.

3.5.1 Frequency distribution of erythrocyte polymorphisms

Glucose-6-phosphate dehydrogenase (G6PD) deficiency was present in 6.1% of the children (Table 3.1).

The frequency of Hb AS (sickle-cell trait) was 7.4 % and the homozygous for HbS was 4.9%. Hb AA also recorded a frequency of 74.4%, whilst the frequency for HbAC was 8.8% (Table 3.2). Alpha+-thalassaemia recorded a frequency of 21.0% for the heterozygous (-α/αα), 8.3% for the homozygous (mutant -α/-α), resulting in a carriage rate (α/αα & -α/-α) of 29.3% (Table 3.3; Figure 3.6). The frequency of *Plasmodium falciparum* parasitaemia was 54.1%.

Table 3.1 Baseline characteristics of G6PD deficiency

	Number	%	Gender (n, % whole sample)		Malaria Parasite (n, % whole sample)	
			Male	Female	+ve	-ve
G6PD						
Normal	1570	93.9	53.7	40.2	842 (50.4)	728 (43.5)
Full Defect	92	5.5	3.3	2.2	56 (3.3)	36 (2.2)
Partial Defect	10	0.6	0.2	0.4	6 (0.4)	4 (0.2)

G6PD is glucose-6-phosphate dehydrogenase. Mps +ve is malaria parasite positive and mps-ve is malaria parasite negative

Table 3.2: Baseline characteristics of β-globin polymorphism

	Number	(%)	Gender (n, % whole sample)		Malaria Parasite (n, % whole sample)	
			Male	Female	+ve	-ve
Hb AA	1247	74.6	42.8	31.8	716 (42.8)	531(31.8)
Hb AS	123	7.4	4.7	2.7	43(2.5)	80(4.8)
Hb AC	147	8.8	5.3	3.5	79(4.7)	68(4.1)
Hb SS	82	4.9	2.6	2.3	38(2.3)	44(2.6)
Hb SC	20	1.2	0.7	0.5	7(0.4)	13(0.8)
Hb CC	1	0.1	0.1	0	0	1(0.1)
Hb AF	44	2.6	0.9	1.7	21(1.3)	23(1.4)
Hb SF	4	0.2	0.1	0.2	1(0.1)	3(0.2)
Hb AD	2	0.1	0	0.1	0	2(0.1)
Hb AFC	2	0.1	0.1	0	0	1(0.1)
Hb ASF	3	0.2	0.2	0	0	3(0.2)

Note: Hb AA-homozygous wild type genotype for β-globin; HbAC- heterozygous haemoglobin C; HbAS- heterozygous sickle cell haemoglobin; HbCC- homozygous haemoglobin C; HbSC- haemoglobin C disease; Hb SS- homozygous for sickle cell disease; G-6-PD- Glucose-6-phosphate dehydrogenase. Mps +ve is malaria parasite positive and mps-ve is malaria parasite negative. **Haemoglobinopathies other than HbAS and HbAC were included in the study because they are not known to protect against severe malaria.**

Table 3.3 Baseline characteristics of α-globin polymorphism

Alpha+-thalassaemia	Number	Gender (n, % mps+ve sample)		Malaria Parasite (n, % mps+ve sample)	
		Male	Female	+ve	-ve
αα/αα	358	34.6	25.7	60.8	-
-α/αα	126	11.2	9.8	21.4	-
-α/-α	49	3.7	4.6	8.3	-
No amplification	56			9.5	-

Note: -α/αα is heterozygous alpha +- thalassaemia; -α/-α is homozygous alpha+- thalassaemia. Mps +ve is malaria parasite positive and mps-ve is malaria parasite negative

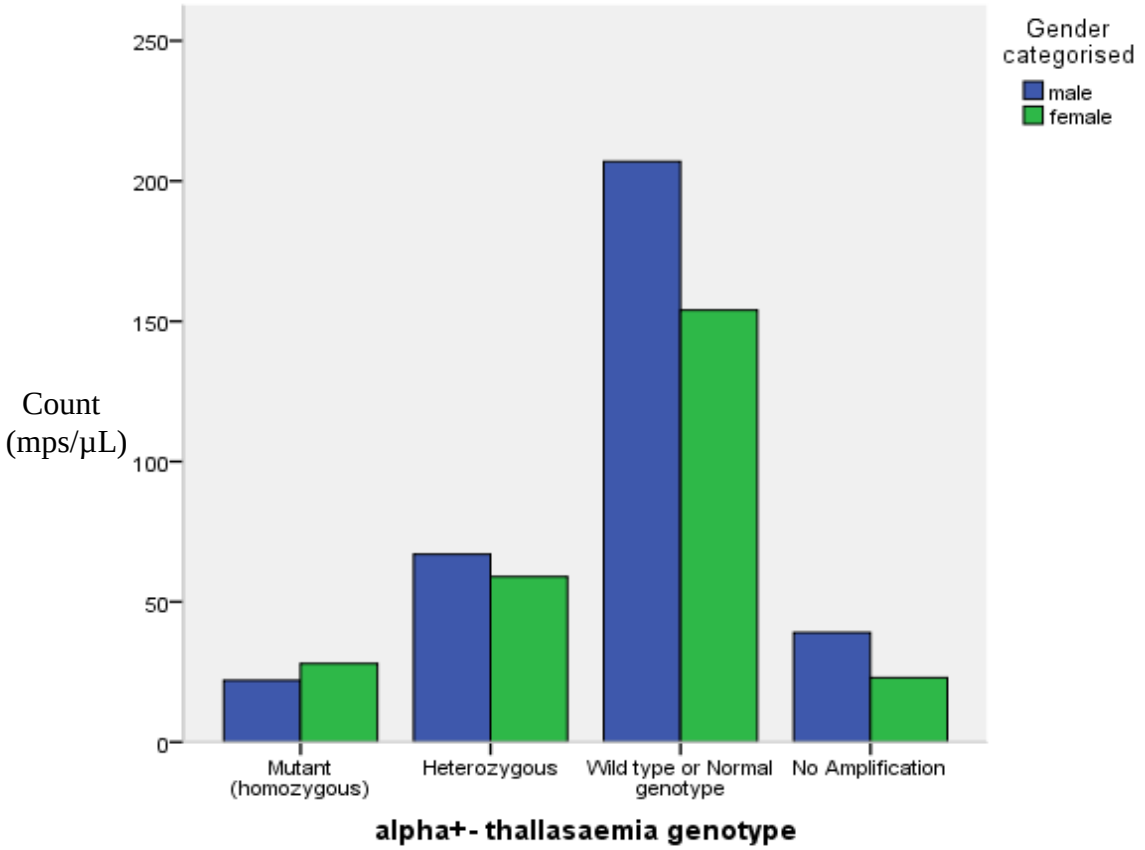

Figure 3.6: Distribution of *Plasmodium falciparum* malaria in alpha+- thalassaemia

3.6 DISCUSSION

The results from this study show that the incidence of malaria infection in the subject population of 1672 children of mean age 51.2 months (range, 1-120 months) is 54.1% indicating that malaria is hyper-endemic in Ghana.

Hitherto, the malaria disease had been considered primarily as a disease of the rural settings, and report by Abdul-Aziz (2006) suggesting approximately 60% of all malaria cases occur among the poorest 20% of the world's population.

Kumasi, however, is an urban settlement in Ghana, and the prevalence rate recorded in this study lends credence to the assertion by Donnelly *et al.,* (2005) that malaria currently presents a leading cause of morbidity and mortality among urban African populations and as indicated by the UNDP report the current rapid growth rate of 3.5% of urban populations in Africa could result in about 50% of African population migrating to urban areas by 2025 (UNPP, 2005).

Previous studies have recorded a prevalence of 8.6% in Accra, the national capital (Klinkenberg *et al.,* 2006), and 13.65% in the Kumasi Metropolis (Asante 2003). Higher incidence rates of 66.90% were reported in northern Ghana by Mockenhaupt *et al.,* (2004a, 2004b), 40% by Amoako *et al*., (2010, personal communication) in the Kintampo North Municipality of Ghana and 25.30% in Kumasi (Ronald *et al*., (2006).

According to Ronald *et al.,* (2006) the socio-economic status of the residents of the Moshie-Zongo and Manhyia communities of Kumasi, Ghana reflected greater exposure to infected mosquitoes through poor quality housing and overcrowding in the communities.

Haemoglobinopathies other than Hb AS, AC trait including G6PD deficient individuals who are known to protect against the severe manifestation of *Plasmodium falciparum* malaria and represent serious confounders in epidemiological studies of alpha+-thalassaemia in Africa, and were excluded from the study (William *et al*., 2005, Wambua *et al*., 2006). As a baseline to this study, the frequencies of these erythrocyte polymorphisms and their susceptibility to *Plasmodium falciparum* malaria parasitaemia was determined.

This study recorded prevalence of 6.1%, 74.4%, 8.8%, and 7.4% for G6PD deficiency, HbAA, HbAC, and HbAS respectively. These compare favourably with HbAS prevalence of 10.9% reported by Kreuels *et al*., (2010), 9.2% by Amoako *et al.,* (2010) and 7% by Mockenhaupt *et al*., (2002).

On the contrary, the World Health Organization had reported 15% prevalence for HbAS (WHO (2006). Kreuels *et al*., (2010) had also reported 10.5% prevalence for HbAC.

The frequency recorded for alpha+-thalassaemia in this study was 21.0% for the heterozygous (-α/αα), 8.3% for the homozygous or mutant (-α/-α), resulting in a carriage rate (α/αα & -α/-α) of 29.3%. All α-globin genotypes were positive for *Plasmodium falciparum* malaria, however the prevalence of malaria was in the order of αα/αα >α/αα >-α/-α.

This result appears to support the basic proposition of Haldane's hypothesis that alpha+-thalassemia is pronounced in malarious regions because it accords carriers a level of protection against death from malaria by assuaging the clinical course of the disease. (Hendricks 2003).

3.7 CONCLUSION

This study has provided the prevalence for the β-globin genotypes, G6PD deficiency and alpha+-thalassaemia among children of the Kumasi metropolitan area. The frequency of *Plasmodium falciparum* malaria was 54.1% indicating that malaria is hyperendemic in Kumasi, an urban population inspite of governmental interventions against mortality due to malaria. The alpha+-thalassaemia also recorded a frequency of 29.3%. Even though alpha thalassaemia does not exclude the presence of iron deficiency, the high prevalence of alpha+-thalassaemia also informs the need to appropriately discriminate iron deficiency from alpha+-thalassaemia to avoid the unnecessary administration of iron therapy.

Chapter 4

4.0 Does microcytosis protect against *Plasmodium falciparum* malaria in alpha+-thalassaemia?

4.1 Introduction

The protective mechanism of alpha+-thalassaemia thus remains unclear with various suggested mechanisms through which alpha+-thalassaemic individuals could confer protection against *Plasmodium falciparum* malaria.

In vitro flow-cytometry studies have shown that erythrocytes of the alpha+ thalassaemia phenotype have reduced parasite growth and/or invasion rate (Pattanapanyasat, *et al.,* 1999) as well as increased binding of antibodies from malaria-immune sera (Williams, *et al.,* 2002) and enhanced splenic clearance of malaria-infected cells. Additionally, thalassaemic RBCs were said to age more quickly due to oxidative stress leading to membrane damage and subsequent increased phagocytosis and an eventual decreased parasite load (Yuthavong *et al.,* 1988; Destro-Bisol *et al.,* 1999 b; Senok *et al.,* 2006).

Red cell rosetting and the sequestration of schizont-infected red cell have been observed in alpha+-thalassaemia individuals in protecting from severe *Plasmodium falciparum* parasitaemia (Carlson *et al.,* 1994). The role of the red cell complement receptor 1 (CR1) associated with rosetting and its reduced expression in alpha+-thalassaemia (both heterozygous and homozygous) allowing protection against severe malaria was observed by Clark, *et al.,* (2003), Cockburn *et al.,* (2004) and Williams, *et al.,* (2005b).

The protective effect by alpha+-thalassaemia against *Plasmodium falciparum* has been suggested to result from modified antigen recognition on the surface of the parasitized erythrocyte and the binding of thalassaemic red cells to increased amounts of immunoglobin and complement (Luzzi *et al.,* 1991a; Luzzi *et al.,* 1991b; Pattanapanyasat *et al.,* 1999; Destro-Bisol *et al*., 1999a, Destro-Bisol *et al.,* 1999b, Cockburn *et al.,* 2004).

Further indications were that these events resulted in the early removal of parasitized cells by the immunoglobin and the sparing of those cells with reduced complement (Cockburn *et al.,* 2004).

Williams *et al.,* (1996), observed that alpha+-thalassaemia is associated with higher levels of *P. vivax* infection early in life which may heighten immunological defences and provided a better protection from subsequent *Plasmodium falciparum* infections.

Higher levels of haptoglobin in the homozygous alpha+-thalassaemic individuals were suggested as toxic to *Plasmodium falciparum in vitro* (Imrie *et al*., 2004), thus conferring protection by removing free haemoglobin released during haemolysis This might explain why alpha+- thalassaemia red cells are apparently less hospitable to parasite growth and multiplication (Imrie *et al*., 2006).

Researchers appear to agree that iron deficiency that results in microcytosis and hypochromasia confers protection from severe manifestation of *Plasmodium falciparum* and that iron is essential for the growth of the parasite (Cheesbrough 1999).

Administration of oral iron-folic acid supplements might therefore increase the risk of morbidity from malaria and/or the bacterial infections that complicate malaria in children (Sazawal *et al*., 2006; WHO, 2006).

Iron deficiency therefore is regarded to influence the course of malaria in *Plasmodium falciparum* infected individuals by offering some protection to the host (Koka *et al*., 2007; Rodak 2002, Cromer *et al*., 2009), via a complex interaction between the parasite, iron status and immune system of the individual Nyakeriga *et al*., (2004b; Sazawal *et al*., 2005).

However, there is still a gap of knowledge regarding the role played by microcytosis in driving protection from the severe manifestations of *Plasmodium falciparum* parasitaemia in alpha+-thalassaemia.

Microcytosis has been shown to be significantly associated with protection from haemoglobin decrease due to *Plasmodium falciparum* and the rate of infection was lower in microcytic than in normocytic anaemia (Mockenhaupt *et al*., 1999b). Fowkes *et al*., (2008) corroborated this finding by indicating that red cell microcytosis was associated with homozygous alpha+-thalassaemia and might be responsible for the protection against severe malarial anaemia.

Recent observation by Vafa *et al.,* (2008) indicated that the altered red cell membrane of alpha+-thalassaemia might affect erythrocyte invasion by *Plasmodium falciparum* with merozoites surface protein (msp2) antigen.

This results in lower susceptibility to infection by certain strains of parasite and reduces the parasite density in these alpha+-thalassaemia individuals with multiple infection.

There are also disagreements between researchers as to the type of alpha+-thalassaemia genotype that confers protection against *Plasmodium falciparum*.

Findings from studies in Papua New Guinea concluded that both heterozygous and homozygous alpha+-thalassaemia protect against severe malaria parasitaemia, though protection was more evident in subjects with homozygous alpha+-thalassaemia (Allen *et al*., 1997). This observation is supported by Williams *et al*., (2005b) who indicated in a Kenya study that both the heterozygous and homozygous alpha+-thalassaemia were protected from severe malaria. In contrast, reports from Tamale, the northern part of Ghana, indicated that alpha+-thalassaemia does protect against death from malaria, however this was only true for heterozygous alpha+-thalassaemia (Mockenhaupt *et al*., 2004a, 2004b).

The inability to detect protection in the homozygous alpha^{+}-thalassaemia might be due to their small number and statistical limitations (Mockenhaupt *et al*., 2004a).

Another Ghanaian study, while agreeing with the ameliorating impact of alpha+-thalassaemia on malaria, concluded that the 'impact' was specific and driven by protection from severe anaemia, the most prevalent malaria complication (May *et al*., 2005). This concept of alpha+-thalassaemia malarial anaemia specificity is supported by another study from Kenya (Wambua *et al*., 2006).

The aims of this study therefore were to test the hypothesis that the protective effect of alpha+-thalassaemia against malarial parasitaemia can be demonstrated in both the heterozygous and homozygous states.

We will also establish whether microcytosis might be responsible for protection from severe malaria parasitaemia amongst alpha+ thalassaemia individuals

4.2 OBJECTIVES

- To compare the geometric mean parasite density between normocytic (control), microcytic, normal genotype (presumed iron deficiency (PID)), homozygous, and heterozygous alpha+-thalassaemia
- Determine the prevalence of different degrees of malaria among normocytic, microcytic, normal genotype (presumed ID) homozygous and heterozygous alpha+-thalassaemia.

4.3 Participants

Seven hundred and thirty-two children in whom *Plasmodium falciparum* malaria were detected (see flow chart, figure 3.1) and who had no confounding factors were tested for haemoglobin (Hb) levels, red blood cell (RBC) count, mean cell volume (MCV), and malaria parasite density. The subjects were then categorised into microcytic and normocytic using an MCV cut-off of 76 fL. Polymerase chain reaction (PCR) for the $-\alpha^{3.7}$ thalassaemia mutation was exclusively done on those subjects that were microcytic (MCV<76 fL).

4.4 Experimental approach

4.4.1 Complete Blood Count (CBC)

Hb, MCV, and RBC counts were estimated following the experimental procedure indicated in section 2.8.2. 3. Microcytosis was defined as MCV (<76 fL).

4.4.2 Polymerase chain reaction (PCR)

The experimental procedures for the extraction of DNA, the polymerase chain reaction and agarose gel electrophoresis are explained in 2.8.3.1

4.4.3 *Plasmodium falciparum* malaria density

Malaria parasite density was determined using the thick and thin blood films by the microscopic method. This followed the experimental procedure as described in section 2.8.1.1

4.5 Statistical analysis

All data were entered in Microsoft excel and analysed using SPSS 17 (IBM Corporation, New York, USA). Statistical analyses were performed as indicated in section 2.9

4.6 RESULTS

Cases were grouped into normocytic (these were control groups whose MCV was >76fL), microcytic (these were children whose MCV was ≤76fL and were not alpha+- thalassaemic and therefore presumed to be iron deficient), homozygous and heterozygous alpha+-thalassaemia based on MCV measurements and PCR. Microcytosis defined as MCV ≤76 fL was initially used to distinguish between normocytic and microcytic children (Menendez *et al*., 2001). PCR was used to discriminate between alpha+-thalassaemia and presumed iron deficient (PID) after excluding confounding factors.

We were unsuccessful in using plasma ferritin at a cut-off of ≤273 μg/L as suggested by Phiri *et al.,* (2009) to provide a 79% specificity and 79% sensitivity in predicting iron deficiency (ID) (Table 4.1). Children who had ferritin levels of ≤273 μg/L were considered as iron deficient whilst those with levels >273 μg/L were regarded as alpha thalassaemic. Contrary to our expectations, we observed that 59/197 of the iron deficient children were alpha thalassaemic suggesting that the ferritin cut off used in this study could not successfully discriminate iron deficiency from alpha thalassaemia. It does also suggest that some of the alpha thalassaemic children were iron deficient, thus corroborating earlier suggestions that iron deficiency might be found among alpha thalassaemias (Imrie *et al*., 2006). This might result from an increased rate of haemolysis in the children leading to a fall in plasma haptoglobin levels and the excretion of free haemoglobin in urine thereby reducing the body's iron stores (Imrie *et al*., 2006).

We performed PCR on the microcytic subjects for the -$\alpha^{3.7}$ deletional type of alpha+-thalassaemia that is mostly found in sub-Saharan Africa and those that were not positive for alpha+ thalassaemias were presumed to be iron deficient.

The association between haematological and parasitological parameters for microcytic, normocytic and alpha+-thalassaemia children as well as the severity of malaria were assessed. The frequency of *Plasmodium falciparum* malaria in the studied population of children who visited the clinic was 54.1%.

4.6.1 Comparism between microcytic and normocytic children in terms of red cell indices and GMPD

There were 589 (80.5%) microcytic children and 143 (19.5%) normocytic children in the study group; MCV (≤76fL) was significantly different between normocytic (MCV, 85.77 ±8.49) and microcytic children (MCV, 70.09 ±5.67) ($p<0.001$). (Table 4.2) Among microcytic children, a significant correlation was observed between MCV and geometric mean parasite density (GMPD) ($r= 0.124$, $p=0.01$, $n=589$) (Figure 4.1). The GMPD however was lower ($p=0.004$) in the normocytic children compared to the microcytic children (Table 4.2).

Haemoglobin concentration was not significantly different between the microcytic and normocytic groups ($p=0.616$), Table 4.2

Table 4.1 Discriminating iron deficiencies from alpha^{+} -thalassaemia amongst microcytic children

		Microcytic children (MCV ≤76 fL) on which ferritin was done (n= 586)		
		Plasma Ferritin		
		≤273 μg/L	**>273 μg/L**	**Total**
N, %		197 (33.62%)	389 (66.38%)	
Alpha^{+} -thalassaemia	-α/αα	19	30	**49**
	-α/-α	40	86	**126**
	αα/αα	117	241	**358**
TOTAL		**176**	**357**	**533**

MCV (mean cell volume); α/αα, heterozygous alpha+- thalassaemia; -α/-α, homozygous alpha+-thalassaemia; αα/αα, normal alpha+- thalassaemia. Children (n=59/197) whose plasma ferritin were ≤273 μg/L were also alpha+-thalassaemic.

Table 4.2 Haematological indices and *Plasmodium falciparum* malaria characterized by microcytosis and normocytic

	Mean (SD)		
	Normocytic (n=143)	Microcytic (n=589)	P-value
Hb (g/dl)	9.01 (3.02)	8.87(2.48)	0.616
RBC (10^6/μL)	3.31(1.14)	3.74(1.06)	<0.001
MCV (fL)	85.77 (8.49)	70.09(5.67)	<0.001
GMPD/μL	**13156(1.31)**	**29087(1.13)**	**0.004**

Independent t-test assessed the differences in the mean values for Hb-haemoglobin, RBC- red blood cell, MCV- mean cell volume, GMPD-geometric mean parasite density. Figures shown are mean and SD

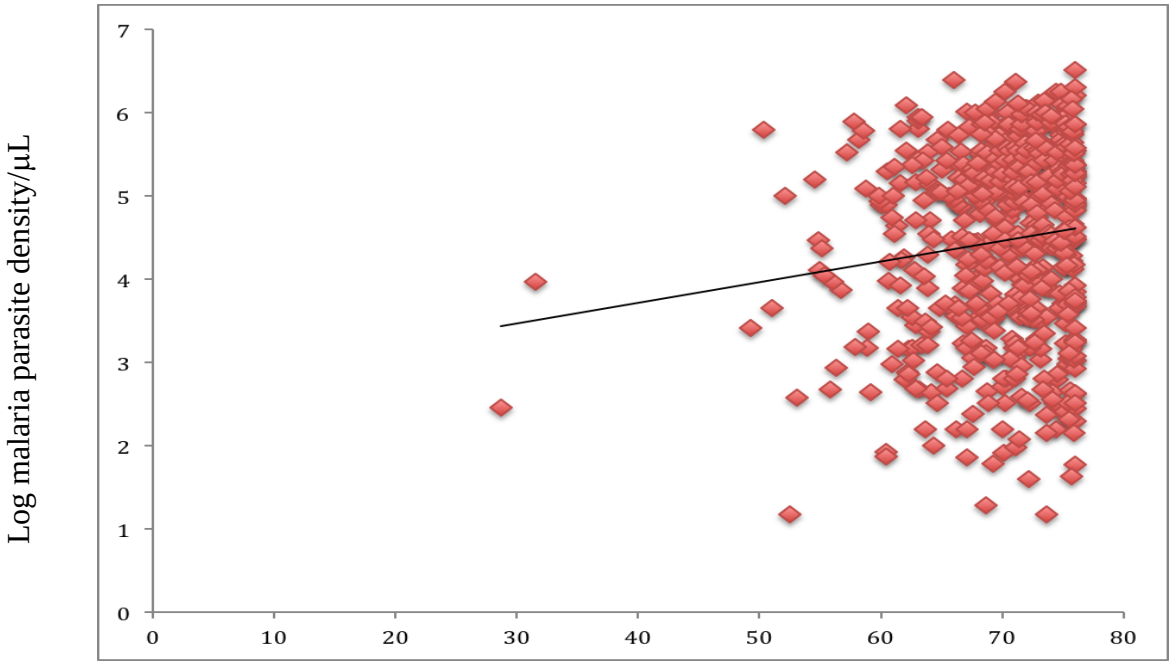

Figure 4.1: Correlation between mean cell volume (MCV) and log malaria parasite density/μL amongst the microcytic

4.6.2 Comparism between alpha globin genotypes in terms of red cell indices and GMPD

Amongst the microcytic population, the -α $^{3.7}$ deletional type of alpha+-thalassaemia recorded a frequency of 21.0% for the heterozygous (-α/αα), 8.3% for the homozygous (-α/-α), resulting in a carriage rate (-α/αα & -α/-α) of 29.3%.

Fifty-six of the subjects did not show any amplification for PCR and were excluded from the analysis.

Both the homozygous and heterozygous alpha+-thalassaemia showed significantly reduced (p <0.001) GMPD compared to normal genotype (presumed iron deficiency-PID) (Table 4.3)

The mean Hb, RBC and MCV significantly differed between the alpha+ thalassaemia genotypes (Tables 4.3). The homozygous alpha+-thalassaemia recorded the lowest haemoglobin concentration

We observe a trend in the association between MCV and GMPD among alpha+-thalassaemia genotypes in the sequence of αα/αα > -α/αα > -α/-α- (Table 4.3). The GMPD values between the alpha+-thalassaemia groups and the normocytic and PID differed significantly (p<0.05) (Table 4.4)

Table 4.3 Haematological indices and *Plasmodium falciparum* malaria characterized by alpha+-thalassaemia genotypes

	Mean (±SD)			
	-α/-α (n=49)	**-α/αα (n=126)**	**αα/αα (PID) (n=358)**	**P-value**
Hb (g/dl)	7.50(2.19)	9.28(2.09)	8.9(2.57)	<0.001
RBC (10^6/μL)	3.39(1.16)	3.97(1.00)	3.65(1.04)	<0.001
MCV (fL)	**68.71(8.31)**	**69.54(5.08)**	**70.88(4.76)**	**0.004**
GMPD/μL	**6852(1.02)**	**9015(0.98)**	**51794(1.10)**	**<0.001**

Analysis of variance (ANOVA) assessed the differences in the mean values Hb-haemoglobin, RBC- red blood cell, MCV- mean cell volume, GMPD-geometric mean parasite density; α/αα, heterozygous alpha +- thalassaemia; -α/-α, homozygous alpha+-thalassaemia; αα/αα, normal or wild type alpha +-thalassaemia; PID=presume iron deficiency. Fifty-six of the samples did not show amplification. Data shown represent mean and SD

Table 4.4: Post-hoc Tukey analysis of means of haematological parameters and malaria parasitaemia by microcytosis

	-α/αα Vrs -α/-α	**-α/-α Vrs αα/αα (PID)**	**-α/αα Vrs αα/αα (PID)**	**Normocytic Vrs -α/-α**	**Normocytic Vrs -α/αα**	**Normocytic Vrs αα/αα (PID)**
Hb (g/dL)	<0.05	<0.05	NS	>0.05	NS	NS
RBC (10^6/μL)	NS	NS	<0.05	NS	<0.05	NS
MCV (fL)	**NS**	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**
GMPD/μL	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**

NS-not significant; Hb-haemoglobin, RBC- red blood cell, MCV- mean cell volume, GMPD-geometric mean parasite density; -α/αα, heterozygous alpha +-thalassaemia; -α/-α, homozygous alpha+-thalassaemia; αα/αα, normal or wild type alpha +- thalassaemia. PID- presumed iron deficient.

4.6.3 The influence of alpha+-thalassaemia on levels of malaria severity in microcytosis

Parasitaemia was expressed as the number of asexual forms of the malaria parasites per microliter and graded as low (1-999/μL) moderate (1000-9999/μL), high (≥ 10000/μL), or severe (≥100000/μL) (Barbara *et al*., 1994). The proportion of patients with severe malaria was reduced in both the homozygous and heterozygous alpha+-thalassaemia groups compared to those with normal genotype (Figure 4.3). Similarly, the proportion of patients with severe malaria was reduced in the alpha+-thalassaemia genotype groups compared to the normocytic, all microcytic and PID groups (Table 4.5)

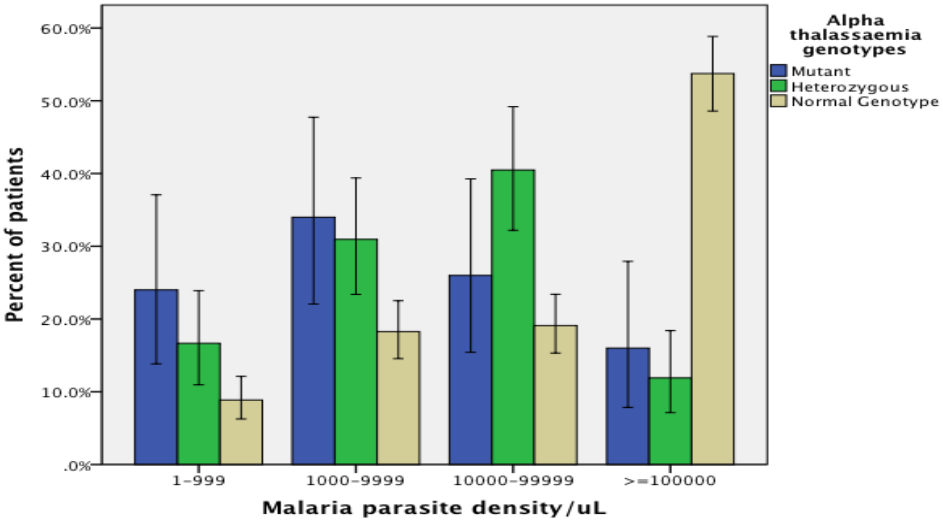

Figure 4.3: Plasmodium falciparum parasitaemia status among alpha+-thalassaemia genotypes (n=533). Error bars: 95% Confidence intreval.Prevalence of severe malaria is significant between alpha+-thalassaemia groups and normal genotypes (p<0.001)

Table 4.5: Incidence of *Plasmodium falciparum* malaria density amongst alpha+-thalassaemia children attending clinic

	Malaria status (n, %)				
Malaria Parasitaemia/μL	Normocytic (n=143)	All Microcytic (n=589)	-α/-α (n=49)	-α/α (n=126)	αα/αα**(PID)** (n=358)
1-999	33 (23.1)	74 (12.6)	12 (24.5)	21 (16.7)	31 (8.7)
1000-9999	40 (28.0)	131 (22.2)	16 (32.7)	39 (31.0)	66 (18.4)
10000-99999	23 (16.1)	140 (23.8)	13 (26.5)	51 (40.5)	69 (19.3)
≥100000	47 (32.9)	244 (41.4)	8 (16.3)	15 (11.9)	192(53.6)*

PID=presume iron deficiency; MPD =mean parasite density; α/αα, heterozygous alpha +- thalassaemia; -α/-α, homozygous alpha+-thalassaemia; αα/αα, normal or wild type alpha +- thalassaemia; Parasitaemia was expressed as the number of asexual forms of the malaria parasites per microlitre graded as low (1-999/μL), moderate (1000-9999/μL), high (10000-99999/μL) and severe (≥100000) n is number of children with various degree of parasitaemia (%). Data shown represents number and percentage of individuals in each group. * p<0.03 by Fisher exact test

4.7 Discussion

The results from this study show that the incidence of malaria infection in the subject population of 1672 children (mean age 51.2 month) is 54.1%. This is slightly lower than incidence rates of 66.90%, reported in Tamale, Northern Ghana (Mockenhaupt *et al*., 2004c), and 77.80% recorded in Southwest Nigeria (Mockenhaupt *et al.,* 1999), which are both areas of intense malaria transmission. The observed incidence of malaria in this study is higher than the 13.65% (range, 2% to 33%) reported by Asante (2003) in a study conducted in the Kumasi Metropolis of Ghana, and the 16.1% incidence rate reported in Ouagadougou, the capital city of Burkina Faso, Sabatinelli *et al.,* (1986).

According to the World Health Organisation (WHO) report of 2010, the incidence of malaria in 2005 to 2009 was 26% -35% in Africa with some countries achieving less than 20% rates.

The prevalence of the -$\alpha^{3.7}$kb type of alpha+-thalassaemia as reported in this study was 21.0% for the heterozygous (-α/αα), 8.3% for the homozygous or mutant (-α/-α), resulting in a carriage rate (α/αα & -α/-α) of 29.3%. This rate is slightly within the 20% to 30% prevalence range in West Africa reported by Yaish (2005). It is, also similar to the 33% (29% Heterozygotes; 4% Homozygotes) prevalence reported by Mockenhaupt *et al.,* (2001) in Agogo, the Ashanti region of Ghana but lower than the 45% reported by Mockenhaupt *et al.,* (1999 b) in Nigeria, or the 53.7% reported by Wambua *et al.,* (2006) on the Coast of Kenya.

Compared to other parts of the world, like the North Coast of Papua New Guinea where the prevalence of the $-\alpha^{3.7}$ deletional alpha+-thalassemia reaches more than 90% (Allen *et al*., 1997) the prevalence rate of alpha+-thalassemia in Ghana, as found in this study, is relatively moderate.

We estimated the prevalence of microcytosis to be 80.5% (mean MCV< 72.6 ±8.86 fL) in the subject population (n=732). Three hundred and fifty eight (48.90%) of the microcytic cases were presumed to be iron deficient, contrary to the 83.5% reported in pre-school children in Ghana (Stanley *et al*., 2001). The high incidence of alpha+thalassaemia amongst the microcytic children does suggest that alpha+-thalassaemia is a key contributor to microcytosis. This finding corroborates reports from Brazil (Borges *et al*., 2001) and Iran (Rahim, 2008) suggesting that the practice of considering iron deficiency as the most common cause of microcytic hypochromic anaemia could prove problematic.

Plasmodium falciparum malaria is the single biggest cause of death in children in Africa and various haemoglobinopathies have been proposed to protect against *the* severe manifestation of malaria. Severe malarial anaemia (SMA) a complicated form of severe anaemia is the leading cause of mortality in malaria-affected children under the age of five years (WHO 2000). One suggestion is that increased microcytosis associated with homozygous $alpha^{+}$-thalassaemia might be responsible for the protection against severe *Plasmodium falciparum* malarial anaemia (Fowkes *et al.,* 2008; Mockenhaupt *et al*., 1999 b).

However, contrary to suggestions by Mockenhaupt *et al*., (1999 b), Fowkes *et al.,* (2008) did not observe any association between parasite load and alpha^{+}-genotype. Some studies have indicated that microcytosis confers protection from malaria in both alpha+-thalassaemia and iron deficiency (Nyakeriga *et al.*, 2004b) and that the mechanism of the protection might be linked to a reduction in the parasite density. Pattanapanyasat *et al.,* (1999) in an *in vitro* study showed that, though normal RBCs and alpha+-thalassaemic RBCs were both susceptible to *Plasmodium falciparum* invasion, the parasite multiplication rates were significantly reduced in the thalassaemic RBC population.

In the opinion of Luzzi *et al*., (1990), red cell disorders in which microcytosis is a characteristic feature contributes to *Plasmodium falciparum* malaria resistance.

We tested the hypothesis that microcytosis might be responsible for protection from severe malaria amongst phenotypically determined alpha^{+}-thalassaemia and non-alpha+ thalassaemia children.

We observed a reduction in the GMPD in normocytic children compared to microcytic children ($p<0.004$, Table 4.2). This is in contrast to observations by Luzzi *et al*., 1990, Mockenhaupt *et al*., (1999 b), and Fowkes *et al.,* (2008) who indicated protection from severe malaria and malarial anaemia in microcytic children who were alpha+-thalassaemia. In this study the microcytic children were heterozygous, homozygous alpha+-thalassaemia and normal genotypes.

However, among the microcytic patients GMPD values were lower in the presence of an alpha-thalassaemia genotype ($p<0.001$, Table 4.2), suggesting that the severity of *Plasmodium falciparum* parasitaemia were significantly lower in both homozygous and heterozygous alpha+-thalassaemia genotypes compared to microcytic children with normal genotype. The protective effect observed in this study was for both heterozygous and homozygous alpha+-thalassaemia but most evident with homozygotes that recorded a significantly lower GMPD compared to both the heterozygotes and normal genotypes ($p< 0.001$).

Our finding conflict with results of a study conducted in Ghana by Mockenhaupt *et al.,* (2004c), which reported that the observed protective effect from *Plasmodium falciparum* malaria was attributable only to the heterozygous form of alpha+-thalassemia. Our finding is however, consistent with Allen *et al.,* (1997) and Williams *et al.,* (2005b) that the protective effect is visible in both genotypes, but most evident in homozygotes.

Severe malaria (defined as MPD >100000/μL) was less prevalent in microcytic patients with an alpha+-thalassaemia genotype (-α/αα 11.9% and -α/-α 16.3%) than either normocytic patients or microcytic patients with a normal genotype (32.9% and 53.6% respectively) ($p<0.03$ by Fisher's exact test). suggesting that both alpha+-thalassaemia genotype were less susceptible to the severe manifestation of malaria and that it is the alpha+-thalassaemia state that confers protection.

Microcytosis (MCV ≤76fL) itself did not influence the incidence of malaria parasite infection as reflected in the proportion of patients in whom parasites were seen, however in the presumed iron deficiency and heterozygous and homozygous alpha+ thalassaemia groups there appeared to be a connection between a progressive decrease in MCV (increased microcytosis) and decrease in parasite density. Both MCV and GMPD were significantly different among these groups (Table 4.3). We also observed a positive correlation among microcytic patients between MCV (Figure 4.1) and malaria parasitaemia and therefore suggest that red cell microcytosis might be associated with a reduction in parasite burden in alpha+ -thalassaemia a view that has been observed by Fowkes *et al*., (2008).

A reduction in parasite density has been indicated in the multiplicity of *Plasmodium falciparum* infection among Senegalese children (Vafa *et al.,* 2008) who observed a correlation between multiple parasite strains and parasite density. They explained, this may be due to the altered membrane of alpha+-thalassaemia red blood cells, which prevented the invasion of *Plasmodium falciparum* with merozoites, surface protein (msp2) and thus reduce the parasite density in these individuals. This study however did not find any evidence of multiple infections among our subject population.

In the current study, we observed in alpha+-thalassaemia children a reduced parasite density, an observation, contrary to the views of (Mockenhaupt *et al.,* (2004a) and Williams *et al.,* (2006) who indicated that the protection by alpha+-thalassaemia did not lower parasite densities.

The present findings are also supported by the suggestion that red blood cells of more severe alpha+thalassaemia genotypes are less hospitable to parasite growth and multiplication (Ifediba *et al*., 1985). By implication the homozygous alpha+-thalassaemia is more protected from severe *Plasmodium falciparum* malaria than the heterozygous alpha+-thalassaemia.

4.8 Summary, limitations and further works

Our study observed a weak but significant correlation between MCV and GMPD among microcytic children. We have also found reduced mean parasite density in both the heterozygous and homozygous alpha+-thalassemia compared to normal genotypes. Alpha+-thalassemia is a key contributor to microcytosis among the study population, accounting for 29.3% in children with microcytosis who might otherwise be regarded as iron deficient. In conclusion, therefore, the severity of *Plasmodium falciparum* parasitaemia measured, as either GMPD or prevalence of severe parasitaemia was significantly lower in both the -α/αα and -α/-α- groups compared to microcytic individuals with normal genotype

Our inability to successfully use ferritin measurements to discriminate microcytosis due to iron deficiency from microcytosis due to alpha+-thalassaemia has been to us a limitation. This would have enabled us to evaluate better the relationship between microcytosis and iron deficiency in protecting against malaria.

Further research might consider enrolling both symptomatic and asymptomatic children for future study. It might also be possible to investigate the epistasis

relationship between some of the haemoglobinopathies and alpha+-thalassaemia in protecting against malaria. The exclusion of subjects with possible inflammation and haemolysis caused by worm infestation other than malaria may address confounding factor issues.

Chapter 5

5.0 Does hypochromasia protect against *Plasmodium falciparum* malaria in alpha^{+}-thalassaemias?

5.1 Introduction

Hypochromasia is a red cell abnormality that results in the decrease in haemoglobin concentration in erythrocytes rendering them pale staining and less intensely than normal. Hypochromasia causes reduction in mean corpuscular haemoglobin (MCH) of red blood cells (Hoffbrand and Moss 2010).

Anaemia and hypochromasia have been observed to be significantly more frequent in alpha+-thalassaemic than in non alpha+-thalassaemic children.

Though the degree to which alpha+-thalassemia contributes to the prevalence of hypochromic anaemia in Africa has not yet been convincingly established, some studies from other parts of the world report that alpha+-thalassemia is a significant contributor to hypochromic anaemia (Rahim, 2008; Borges *et al*., 2001; Sankar *et al*., 2006).

One of the features of alpha+-thalassaemia is haemolysis. Generally, both heterozygous and homozygous alpha+-thalassaemia have lower levels of total Hb, mean cell haemoglobin concentration, mean corpuscular volume, and mean cell haemoglobin concentration than normal alpha^{+}-thalassaemias (αα / αα)**.**

However, there is a greater difference between heterozygotes and homozygotes than between heterozygotes and normal alpha^{+}-thalassaemias (Imrie *et al.*, 2006). Therefore, compared to normal alpha^{+}-thalassaemias, heterozygotes do not present any clinical symptoms, whereas homozygotes are characterized by mild hypochromic and microcytic anaemia (Imrie *et al.*, 2004).

Reports that Hb levels are further lowered in iron depleted alpha+-thalassaemic children suggest that inhibition of α-globin synthesis might be responsible (Mockenhaupt *et al*., 1999).

Malaria associated anaemia was observed to be less pronounced among homozygous alpha^{+}- thalassaemia children than in the normal genotype. However, observations by Veenemans *et al*.,(2008) showed that alpha+-thalassaemia actually limits the decline in haemoglobin concentration associated with afebrile *Plasmodium falciparum* infection. The effect of alpha^{+}-thalassaemia on haemoglobin level was observed among Tanzanian children with uncomplicated malaria (Enevold *et al.,* 2008) where alpha^{+}-thalassaemias were found to be associated with reduced haemoglobin levels. In the opinion of Pasvol, (2006) whilst haemoglobin concentrations were lower among alpha^{+}-thalassaemia children in steady state and during mild *Plasmodium falciparum* attack, haemoglobin concentrations were relatively higher in children with severe malaria.

A similar observation has been made in Kenyan children who were both heterozygous and homozygous for alpha^{+}-thalassaemia (Wambua *et al*., 2006) suggesting that alpha+-thalassemia may protect against the progression of anaemia during the course of severe manifestation of *Plasmodium falciparum* parasitaemia. Among children with acute *Plasmodium falciparum* malaria haemoglobin concentrations were lower in normal alpha+-thalassemia genotypes than were found in both the heterozygous and homozygous alpha+-thalassemia (Fowkes *et al*., 2008). However, Hb concentrations were higher in the homozygous than in the heterozygous alpha+-thalassemia.

5.2 OBJECTIVES

- To compare the geometric mean parasite density (GMPD) between normochromic (control), hypochromic, normal genotype (presumed iron deficiency (PID), homozygous and heterozygous alpha+-thalassaemia
- Determine the prevalence of different degrees of malaria among normochromic, hypochromic, normal genotype, (presumed ID) and homozygous and heterozygous alpha+-thalassaemia.

5.3 Participants

Refer to section 2.1 and flow chart, figure 3.2 of chapter 3

5.4 Experimental approach

5.4.1 Complete Blood Count (CBC)

Hb, MCH, and RBC counts were estimated following the experimental procedure indicated in section 2.8.2.3.

5.4.2 Polymerase chain reaction (PCR)

The experimental procedures for the extraction of DNA, the polymerase chain reaction and agarose gel electrophoresis are explained in 2.8.3.1

5.4.3 *Plasmodium falciparum* malaria density

Malaria parasite density was determined following experimental procedure as describe in section 2.8.1.1

5.5 Statistical analysis

All data were entered in Microsoft excel and were analysed using SPSS 17 (IBM Corporation, New York, USA) as indicated in section 2.9.

5.6 RESULTS

There were 267 (36.5%) hypochromic children and 465(63.5%) normochromic children in the study group. Cases were grouped into normochromic (control), hypochromic, with normal genotype (presumed iron deficiency (PID), and homozygous and heterozygous alpha+-thalassaemia based on PCR. MCH cut-off of ≤25 pg was used to discriminate normochromic from hypochromic children.

The association between haematological and parasitological parameters for hypochromic children as well as the severity of malaria were assessed.

5.6.1 Comparism between hypochromic and normochromic children in terms of red cell indices and GMPD

Haemoglobin concentration was not significantly different between normochromic and hypochromic children (p=0.481, Table 5.1) however the difference in the red blood cell count values was significant (p<0.001).

The GMPD in the hypochromasia was significantly higher than that in normochromic children (p<0.001) suggesting that hypochromic children were more susceptible to *Plasmodium falciparum* infection than the normocytic children (Table 5.1). The correlations of *Plasmodium falciparum* parasite density with haemoglobin and MCH concentrations were not significant among the hypochromic children (r=0.21, p=0.653, n=267, and r=0.053, p=0.474, n=267, respectively (Figures 5.1 and 5.2), as well as normochromasia group (r=0.030, p=0.623, n=465).

Table 5.1: Haematological indices and *Plasmodium falciparum* malaria characterized by hypochromasia and normochromasia

	Mean (SD)		
	Normochromic N=465	**Hypochromic N=267**	**P-value**
Hb (g/dl)	8.95(2.67)	8.81(2.44)	0.481
RBC (10^6/μL)	3.51 (1.07)	3.91(1.78)	<0.001
MCH (pg)	**25.76(3.86)**	**22.70(2.30)**	**<0.001**
GMPD/μL	**22134(1.20)**	**30603(1.14)**	**<0.001**

Independent t-test assessed the differences in the mean values
Hb-haemoglobin, RBC- red blood cell, MCH- mean corpuscular haemoglobin, GMPD-geometric mean parasite density

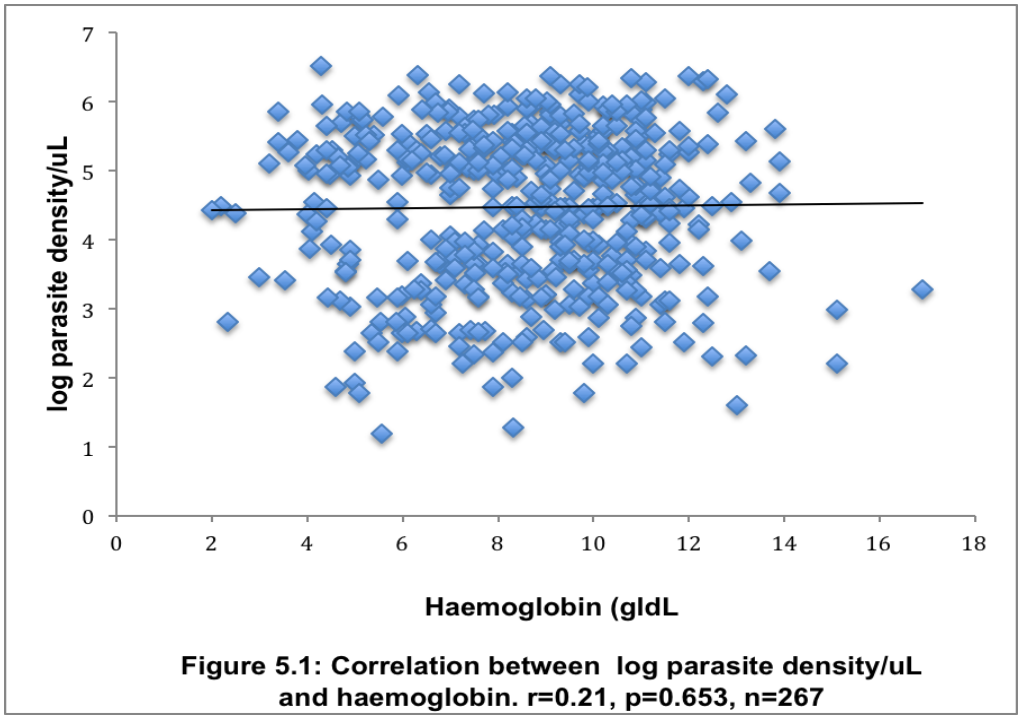

Figure 5.1: Correlation between log parasite density/uL and haemoglobin. r=0.21, p=0.653, n=267

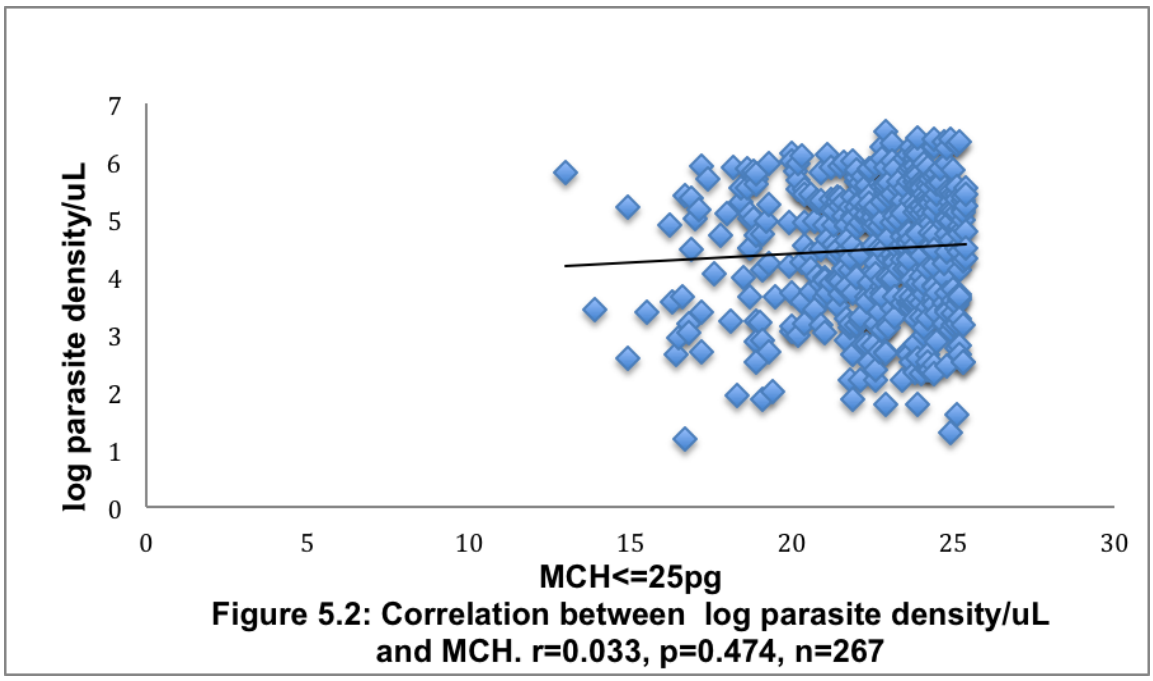

Figure 5.2: Correlation between log parasite density/uL and MCH. r=0.033, p=0.474, n=267

5.6.2: Comparism between alpha globin genotypes in terms of red cell indices and GMPD

In all the haematological and parasitological tests, comparisons between the alpha +-thalassaemia genotypes, showed significant differences for only MCH and GMPD. GMPD was comparatively low in the homozygous compared to the heterozygous alpha+-thalassaemia genotypes, and the normal or presumed iron deficiency (Table 5.2). Haemoglobin values were largely depended on genotype (normal, 8.78 g/dL; heterozygous, 9.17 g/dL; and homozygous, 8.06 g/dL). Mean haemoglobin concentration was not significantly different amongst all α-globin genotypes (Table 5.2).

Haemoglobin value did not correlate with mean parasite density (MPD) (r=0.21, p=0.653 Figure 5.1) and does not appear influence parasitaemia in alpha +thalassaemia.

Table 5.2: Hypochromasia and *Plasmodium falciparum* malaria characterized by alpha+-thalassaemia genotypes

	Mean (SD)			
	-α/-α (n=23)	**-α/αα (n=44)**	**αα/αα (PID) (n=141)**	**P-value**
Hb (g/dl)	8.06(2.22)	9.17(1.89)	8.78(2.53)	0.075
RBC (10^6/μL)	3.77(1.03)	4.08(0.95)	3.89(1.10)	<0.458
MCH (pg)	**21.45(2.94)**	**22.55(2.08)**	**22.81(2.20)**	**<0.029**
GMPD/μL	**7160(1.00)**	**17280(0.89)**	**48997(1.09)**	**<0.001**

Analysis of variance (ANOVA) assessed the differences in the mean values Hb-haemoglobin, RBC- red blood cell, MCH- mean corpuscular haemoglobin, GMPD-geometric mean parasite density; α/αα, heterozygous alpha +-thalassaemia; -α/-α, homozygous alpha+-thalassaemia; αα/αα, normal or wild type alpha +- thalassaemia; PID=presume iron deficiency. Data represents mean and SD

Table 5.3: Post-hoc Tukey analysis of means of haematological parameters and malaria parasitaemia by hypochromasia

	-α/αα Vs. -α/-α	**-α/-α Vs. αα/αα (PID)**	**-α/αα Vs. αα/αα (PID)**	**Normochromic Vs. -α/-α**	**Normochromic Vs. -α/αα**	**Normochromic Vs. αα/αα (PID)**
Hb (g/dL)	NS	NS	NS	NS	NS	NS
RBC (10^6/μL)	NS	NS	NS	NS	<0.05	<0.05
MCH (pg)	**NS**	**<0.05**	**NS**	**NS**	**<0.05**	**<0.05**
GMPD/μL	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**

Hb-haemoglobin, RBC- red blood cell, MCH- mean corpuscular haemoglobin, GMPD-geometric mean parasite density, α/αα, heterozygous alpha +-thalassaemia; -α/-α, homozygous alpha+-thalassaemia; αα/αα, normal or wild type alpha +- thalassaemia. NS-not significant; PID-presumed iron deficiency

5.7: Discussion

The primary objective of this study was to determine whether hypochromic red blood cells (MCH ≤25 pg) are responsible for conferring protection from the severe manifestation of *Plasmodium falciparum* malaria in alpha+ thalassaemias. Of particular interest to us is whether the said protection might result in reducing the parasite density in the alpha+ -thalassaemic patients.

Very little is however known of the role of hypochromasia, which is the decrease in haemoglobin concentration in erythrocytes in conferring protection from severe *Plasmodium falciparum* malaria in alpha+ thalassaemias.

The scarcity of haemoglobin has been suggested to provide a non-conducive nutrient-deficient environment for *Plasmodium falciparum* growth (Nyakeriga *et al*., 2004 b).

In the hypochromic children, the haemoglobin level did not differ significantly ($p=0.481$) from the normocytic children even though the level in normocytic children appeared slightly higher compared to haemoglobin level in the hypochromic children. This might explain the significantly high ($p<0.001$) GMPD observed in the hypochromic children compared to the normocytic children due to haemoglobin decline. This not withstanding, we did not find any evidence to suggest a relationship between *Plasmodium falciparum* parasitaemia and haemoglobin (Figure 5.1)

Both heterozygous and homozygous alpha+-thalassaemia have been implicated in preventing the decline in haemoglobin concentration in children with clinical malaria (Wambua *et al*., 2006). We observed haemoglobin levels to be independent of heterozygous, homozygous and normal alpha^{+}-thalassaemia genotypes however children heterozygous for alpha^{+}-thalassaemia had higher mean haemoglobin concentration than did children with normal genotype. This suggests that alpha^{+}-thalassaemia might prevent the decline in haemoglobin concentration in the heterozygous for alpha^{+}-thalassaemia.

This compares to other works that have suggested that alpha+ thalassaemia might limit the decline in haemoglobin that is not only confined to severe malaria cases but even so the protective effect might be evident when the *Plasmodium falciparum* infection is accompanied by inflammation (Veenemans *et al*., 2008).

But the fact that GMPD in the hypochromic differed significantly from the normochromic group ($p<0.001$), recording an increase in the density of parasite compared to the normocytic group suggests that hypochromic alpha+ thalassaemia were not biologically advantageous in terms of protection from parasitaemia over their normochromic counterparts. Hypochromic patients were therefore more susceptible to *Plasmodium falciparum* parasitaemia than the normochromic counterparts. This is however in contrast to suggestions that the scarcity in haemoglobin might provide an environment conducive for the *Plasmodium falciparum* malaria to thrive. Relative to children with normal alpha^{+}-thalassaemia genotype, GMPD differed significantly ($p<0.001$) compared to both the heterozygous and homozygous alpha^{+}-thalassaemias suggesting that protection from the severe manifestation of *Plasmodium falciparum* parasitaemia might not be due hypochromic red cells per se but rather alpha^{+}-thalassaemia genotypes who are hypochromic.

In addition we observed a progressive decrease in MCH compared to GMPD (Table 5.2) in the order of homozygous <heterozygous <normal alpha^{+}-thalassaemias, a trend that was observed among the microcytic children with alpha^{+}-thalassaemia.

This trend might explain the fact that both microcytic and hypochromic are inherited together in either iron deficiency anaemia or the thalassaemias and that the protection against severe malaria in heterozygous and homozygous alpha^{+}-thalassaemias who are microcytic might be implicated in hypochromic alpha+-thalassaemias.

5.8: Summary, limitations and further works

Even though GMPD and MCH differed significantly among all alpha^{+}-thalassaemia genotypes, our data did not observe any significant correlation between hypochromasia and *Plasmodium falciparum* parasitaemia. Hypochromasia therefore might not confer protection from the severe manifestation of *Plasmodium falciparum* parasitaemia in alpha+-thalassaemia genotypes and did not prevent infection.

The study also found that haemoglobin levels were independent of GMPD and that alpha^{+}-thalassaemia genotype did not influence a decline in haemoglobin concentration.

Further works might have to discriminate other causes of hypochromic and microcytic anaemia like worm infestation which is common among the population and the possible interaction between other haemoglobinopathies like HbSC and HbSS that causes haemoglobin reduction and therefore hypochromasia.

Chapter 6

6.0 Does severe malarial anaemia protect against *Plasmodium falciparum* malaria in alpha^{+}-thalassaemias?

6.1 Introduction

Severe malarial anaemia (SMA) is the leading cause of mortality in malaria-affected children under the age of five years (WHO 2000). In acute childhood malaria, suppression of bone marrow responsiveness and the increased phagocytosis of parasitized red blood cells (pRBC) and non-parasitized red blood cells (n-pRBC) play a major role in SMA (Ekvall, 2003). Both haemolysis and suppression of normal erythropoiesis have been indicated to cause SMA in children with severe *Plasmodium falciparum* parasitaemia (Menendez *et al*., 2000).

Indications from a Ghanaian study were that the protection against malaria death in alpha+-thalassaemia was specific and driven by protection from severe anaemia, the most prevalent malaria complication (May *et al.*, 2005). Among a cohort of Gambian children heterozygous and homozygous for alpha+-thalassaemia, Allen *et al*., (1997) observed protection against SMA.

This concept of alpha+-thalassemia's anaemia dependent protection against malarial complications is supported by another study from Kenya (Wambua *et al.*, 2006) where it is suggested that the protection might be as a result of a promoter polymorphism of red cell complement receptor 1 (CR1), for red cell rosetting. Alpha^{+}-thalassaemia has been implicated to independently associate with reduced expression of red cell CR1 and thus reduce the ability of red cells to form rosettes, (Clark, *et al.,* 2003, Williams, *et al.,* 2005a) leading to protection against malarial complications

In the opinion of Veenemans *et al*., (2008) alpha^{+}-thalassaemia confers protection against SMA in children by preventing a gradual decline in haemoglobin concentration during repeated or chronic infection.

It has been suggested that not only did homozygous alpha^{+}-thalassaemia protect from severe forms of *Plasmodium falciparum* malaria but also protect against SMA (Wambua *et al*., 2006, William *et al*., 2005, Mockenhaupt *et al*., 2004, May *et al*., 2007); that the mechanism for this protection against SMA might be an increased in erythrocyte count and microcytosis thus maintaining the Hb concentration above 5.0g/dL (Fowkes *et al*., 2008). Evidence of this protection has been observed among Papua New Guinean children who were homozygous for alpha+-thalassaemia (Fowkes *et al*., 2008).

This notwithstanding, there is still a gap of knowledge regarding the mechanism by which alpha^{+}-thalassaemia confer protection from SMA.

However, with reports that microcytosis and hypochromic erythrocytes reduce the decline in haemoglobin concentration, Danquah and Mockenhaupt (2008) have suggested that alpha^{+}-thalassaemia might protect against SMA during acute and chronic *Plasmodium falciparum* parasitaemia and thus reduce the risk of anaemia.

6.2 OBJECTIVES

- To determine the prevalence of severe anaemia (Hb<5 g/dL) in hypochromic, normal genotype (presumed ID) homozygous and heterozygous alpha+-thalassaemia
- Determine whether severe malarial anaemia drives protection from *Plasmodium falciparum* parasitaemia

6.3 Participants

Refer to section 2.1 and flow chart, figure 3.2 of chapter 3

6.4 Experimental approach

6.4.1 Complete Blood Count (CBC)

Hb, MCH, and RBC counts were estimated following the experimental procedure indicated in section 2.8.2.3.

6.4.2 Polymerase chain reaction (PCR)

The experimental procedures for the extraction of DNA, the polymerase chain reaction and agarose gel electrophoresis are explained in 2.8.3.1

6.4.3 *Plasmodium falciparum* malaria density

Malaria parasite density was determined following experimental procedure as describe in section 2.8.1.1

6.5 Statistical analysis

All data were entered in Microsoft excel and were analysed using SPSS 17 (IBM Corporation, New York, USA) as indicated in section 2.9

6.6 RESULT

6.6.1 Severe malarial anaemia in alpha+ thalassaemia genotypes

Severe anaemia was defined as Hb<5 g/dL (WHO, 2000).

Severe malarial anaemia was associated with higher parasite density in heterozygous and homozygous alpha+ thalassaemia as children with severe anaemia had higher GMPD compared to those whose haemoglobin values were more than 5 g/dL (Table 6.1). The proportions of severe anaemia cases associated with *Plasmodium falciparum* malaria infection were largely independent of all alpha+ thalassaemia genotypes (Table 6.1).

Table 6.1: Prevalence of severe malarial anaemia and MPD among alpha+-thalassaemia genotypes

	Severe malarial anaemia (n,%)		GMPD (SD)
-α/-α	≤ 5 g/dL	9 (18.0)	17725(5.37)*
	>5 g/dL	40 (82.0)	5387(5.08)
-α/α	≤ 5 g/dL	7 (5.6)	12040(5.51)*
	>5 g/dL	119 (94.4)	8863(4.95)
αα/αα (PID)	≤ 5 g/dL	32 (8.9)	32732(5.76)*
	>5 g/dL	325 (90.5)	55552(5.59)

PID=presumed iron deficiency; GMPD = geometric mean parasite density; -α/αα, heterozygous alpha^{+}-thalassaemia; -α/-α, homozygous alpha^{+}-thalassaemia; αα/αα, normal or wild type alpha^{+}- thalassaemia; Severe malarial anaemia is defined as haemoglobin concentration ≤ 5 g/dL. *The comparism between the GMPD of severe anaemia and non-severe anaemia is significant at p<0.001

6.7 Discussion

We determined the influence of SMA on *Plasmodium falciparum* parasitaemia. Of particular interest to us is whether SMA might result in reducing the parasite density in the alpha^{+}-thalassaemic patients.

Some of the mechanisms that have been suggested to protect from the severe manifestation of *Plasmodium falciparum* malaria have been the protection from severe anaemia (May *et al.,* 2005). Malaria generally causes haemolysis and therefore a reduction in the haemoglobin concentration. However, among alpha^{+}-thalassaemic patients is a gradual decline in the haemoglobin concentration (Veenemans *et al*., 2008) thus conferring protection against SMA in children.

Our finding suggests that children with SMA were not advantageous in protecting from severe malaria but rather had increase *Plasmodium falciparum* parasitaemia as shown in table 6.1. Our data therefore suggests that the protection conferred by alpha^{+}-thalassaemias is confined to children that had no SMA and whose haemoglobin concentration was ≤ 5 g/dL

The observation that alpha^{+}-thalassaemias were protected from severe malaria only when there was severe anaemia (Pasvol, 2006) was not evident in our study. We defined SMA as Hb ≤5 g/dL in association with parasite density ≥100000/µL.

Amongst the homozygous and heterozygous alpha+-thalassaemia, children with SMA had a significantly high GMPD than their counterparts who were not severely anaemic ($p<0.001$, Table 6.1).

Our finding is however consistent with previous observation by May *et al.,* (2005) who indicated that the protection from malaria by alpha^{+}-thalassaemia was driven by protection from severe anaemia. In other words severe anaemia did not offer any protection against *Plasmodium falciparum* parasitaemia in alpha^{+}-thalassaemia genotypes.

The proportion of SMA in the homozygous and heterozygous alpha^{+}-thalassaemia groups was less compared to the normal genotype and this might explain the limit in the decline of haemoglobin concentration in alpha^{+}-thalassaemia associated with malaria (Veenemans *et al*., 2008). We are therefore of the opinion that alpha^{+}-thalassaemia genotypes might protect from Hb decline, however this protection could wane should the Hb concentration fall below 5 g/dL. This is because in children with severe *Plasmodium falciparum* parasitaemia, phagocytosis of both parasitized and non-parasitized red blood cells as well as haemolysis are increased resulting in SMA (Ekvall, 2003).

6.8: Summary, limitations and further works

A major outcome of this study is the observed relationship between SMA and GMPD in the alpha+-thalassaemia genotypes where SMA might be implicated in increased GMPD among heterozygous and homozygous alpha^{+}-thalassemia. We were unable to determine the reason for the loss of protection from severe *Plasmodium falciparum* parasitaemia in children who were severely anaemic and future works might examine the mechanism responsible for protecting alpha^{+}-thalassemia children who were not severely anaemic from severe *Plasmodium falciparum* parasitaemia.

Chapter 7

7.0 The waning of protection from *Plasmodium falciparum* in alpha^{+}-thalassaemia

7.1 Introduction

The prevalence of alpha^{+}-thalassaemia trait in Tamale, Ghana and its surroundings was estimated to be 31.8% with the homozygous alpha^{+}-thalassaemia recording prevalence rate of between 2-3% (Hendricks 2003, Mockenhaupt *et al.*, 2004c). The protective effects of alpha^{+}-thalassaemia trait against severe malaria have been demonstrated mostly amongst children $\leq$10 years (Mockenhaupt *et al.*, 2004c)

The protective effects of the sickle cell trait (HbAS) against severe and uncomplicated *Plasmodium falciparum* malarial infection have been reported in Kenya (Aidoo *et al.,* 2002) where they are restricted to younger children below 5 years. William *et al.,*(1996) suggested that the incidence of uncomplicated malaria (of *Plasmodium vivax*) amongst alpha^{+}-thalassaemia children was significantly higher in younger children than in older children. Similarly, the protective effect from malaria-associated mortality in heterozygous alpha^{+}-thalassaemia is said to be strongest in younger children (Mockenhaupt *et al.*, 2004c) with the explanation that innate resistance will normally act at an early age before specific immunity develops at a later age.

Luzzi and his colleagues have also indicated that the incidence of *Plasmodium vivax* infection was increased in children aged less than 30 months (Luzzi *et al*., 1997). In the opinion of Allen *et al.,* (1997), homozygous alpha^{+}-thalassaemia children of a median age of 3.6 years had a tendency towards reduced malaria; he further explained that the exposure to malaria infection and subsequently the intensity of the malaria parasitaemia determines the age at which clinical immunity develops. However, reports from Melanesia have indicated that homozygous alpha^{+}-thalassaemia children of less than 5 years old had twice the incidence of *Plasmodium falciparum* or *Plasmodium vivax* (William *et al.,* 1996) with the suggestion that an increased susceptibility to malaria in infancy might result in improved immunity to subsequent severe parasitaemia by enhanced antigen expression on *Plasmodium falciparum*–infected alpha^{+}-thalassaemic erythrocytes. In support of this observation, Enevold *et al.,* (2008), who also investigated the risk of uncomplicated malaria in alpha+ thalassaemia children observed that the protective effect was prominent in both heterozygous and homozygous alpha^{+}-thalassaemia who were older than 5 years old.

Thus there is still a gap of knowledge as to whether the protection conferred by alpha^{+}-thalassaemia wanes with age. The aim of this study is to determine whether the protective effect conferred by alpha^{+}-thalassaemia against *Plasmodium falciparum* parasitaemia wanes with age

7.2 Objectives

- To compare the proportion of patients with various levels of *Plasmodium falciparum* (1-9999, 10000-99999 and ≥100000) parasitaemia in children >60 months and ≤60 months age groups for the alpha^{+}-thalassaemia genotypes
- To compare the mean parasite density in various alpha^{+}-thalassaemia genotype groups for children ≤60 months and >60 months of age.

7.3 Participants

Participants were five hundred and thirty-three children who were positive for *Plasmodium falciparum* malaria and were 120 months and below. They constitute, heterozygous (n=126), homozygous (n=49) alpha^{+}-thalassaemia and normal genotypes (n=358). The children were grouped into two age categories of ≤ 60 months and >60 months.

7.4 Experimental approach

7.4.1 Polymerase chain reaction (PCR)

The experimental procedures for the extraction of DNA, the polymerase chain reaction and agarose gel electrophoresis are explained in 2.8.3.1

7.4.2 *Plasmodium falciparum* Malaria Density

Malaria parasite density was determined using the thick and thin blood films by the microscopic method. This followed the experimental procedure as described in section 2.8.1.1

7.5 Statistical analysis

The proportion of severe malaria (defined as parasite density ≥100000 parasite/μL) in the age group ≤ 60 months amongst the alpha globin genotypes was determined and compared with the age group >60 months using the Fisher's exact test. Geometric Mean Parasite Density (GMPD) was determined after the log transformation of the values malaria parasite density.

7.6 Results

Cases were grouped into ages ≤ 60 months and >60 months. The mean age overall was 51.2 months (range 1-120 months). A total of 215 cases of severe malaria (≥100000 parasite/μL) were recorded in the alpha^{+}-thalassaemia genotypes (homozygous-8, heterozygous-15 and normal genotype-193) (Table 7.1). All children had *Plasmodium falciparum* malaria.

The distribution of severe malaria parasitaemia amongst the various age groups was determined. The density of parasitaemia in the age groups for alpha^{+}-thalassaemia genotypes was also assessed.

7.6.1: Comparism of *Plasmodium falciparum* parasitaemia between age group ≤60 months and >60 months amongst the alpha^{+}-thalassaemia genotypes.

The proportion of severe malaria cases amongst children ≤60 months and >60 months who were either homozygous or heterozygous for alpha^{+}-thalassaemia was assessed. This study found that whilst there were more severe malaria cases in children ≤60 months compared to the children >60 months who were homozygous and heterozygous for alpha^{+}-thalassaemia (Table 7.1), there were no statistical difference between these groups (p=0.8281 and p=0.7412 respectively). The differences in the mean parasite densities between the two age groups for homozygous and heterozygous alpha^{+}-thalassaemia were not significant for the various degree of parasitaemia (1-9999, p=0.502; 10000-99999, p=0.428; ≤100000, p=0.399, Table 7.1). Even though the geometric mean parasite density (GMPD) of both the homozygous and heterozygous alpha^{+}-thalassaemia were significantly different (p<0.001) within the individual age groups of ≤60 months and >60 months (Table 7.2), the differences in the GMPD between the two age groups for both the homozygous and heterozygous alpha^{+}-thalassaemia were not significant (p=0.207, Figure 7.2).

Table 7.1: Percentage distribution of *Plasmodium falciparum* parasitaemia amongst age groups of alpha^{+} thalassaemia children

Age Group (month)		alpha^{+} thalassaemia	Mean parasite density/μL, n (%)			Total
			1-9999	10000-99999	≥100000	
0-12		-α/-α	3(75.0)	1(25.0)	0	4
		-α/αα	10(50.0)	7(35.0)	3(15.0)	20
		αα/αα	10(23.8)	9(21.4)	23(54.8)	42
	Total		**23**	**17**	**26**	**66**
13-24		-α/-α	7(50.0)	3(21.4)	4(28.6)	14
		-α/αα	16(59.3)	9(33.3)	2(7.4)	27
		αα/αα	28(35.4)	10(12.7)	41(51.9)	79
	Total		**51**	**22**	**47**	**120**
25-36		-α/-α	10(76.9)	2(15.4)	1(7.7)	13
		-α/αα	12(48.0)	10(40.0)	3(12.0)	25
		αα/αα	10(16.7)	17(28.3)	33(55.0)	60
	Total		**32**	**29**	**37**	**98**
37-48		-α/-α	1(20.0)	2(40.0)	2(40.0)	5
		-α/αα	6(35.3)	10(58.8)	1(5.9)	17
		αα/αα	15(29.4)	8(15.7)	28(54.9)	51
	Total		**22**	**20**	**31**	**73**
49-60		-α/-α	1(33.3)	2(66.7)	0	3
		-α/αα	3(27.3)	4(36.4)	4(36.4)	11
		αα/αα	5(19.2)	3(11.5)	18(69.2)	26
	Total		**9**	**9**	**22**	**40**
61-72		-α/-α	2(50.0)	1(25.0)	1(25.0)	4
		-α/αα	4(66.7)	1(16.7)	1(16.7)	6
		αα/αα	6(19.4)	8(25.8)	17(54.8)	31
	Total		**12**	**10**	**19**	**41**

Table 7.1 continued

Age Group (month)		alpha^{+} thalassaemia	Mean parasite density/μL, n (%)			Total
			1-9999	10000-99999	≥100000	
73-84		-α/-α	1(50.0)	1(50.0)	0	2
		-α/αα	0	3(100)	0	3
		αα/αα	8(40.0)	3(15.0)	9(45.0)	20
	Total		**9**	**7**	**9**	**25**
85-96		-α/-α	1(100)	0	0	1
		-α/αα	4(50.0)	4(50.0)	0	8
		αα/αα	10(40.0)	5(20.0)	10(40.0)	25
	Total		**15**	**9**	**10**	**34**
97-108		-α/-α	2(100)	0	0	2
		-α/αα	3(50.0)	2(33.3)	1(16.7)	6
		αα/αα	1(12.5)	2(25.0)	5(62.5)	8
	Total		**6**	**4**	**6**	**16**
109-120		-α/-α	1(50.0)	1(50.0)	0	2
		-α/αα	2(66.7)	1(33.3)	0	3
		αα/αα	4(23.5)	4(23.5)	9(52.9)	17
	Total		**7**	**6**	**9**	**22**

Severe malaria is parasite density ≥ 100000/μL. n is number of children with severe *Plasmodium falciparum* parasitaemia. Homozygous alpha+-thalassaemia (-α/-α), Heterozygous alpha+-thalassaemia (-α/αα), Normal genotype (αα/αα)

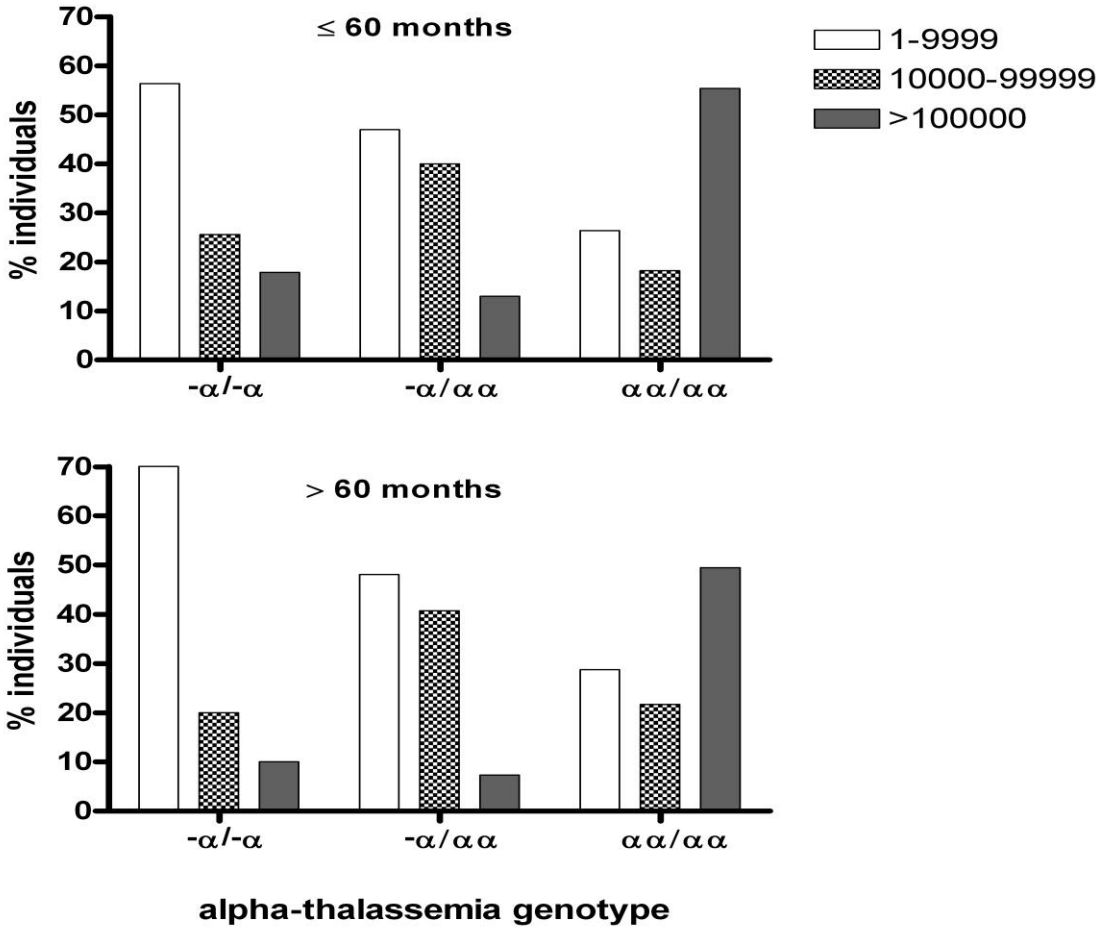

Figure 7.1: Distribution of patients in each category of *Plasmodium falciparum* malaria parasite density of the various age groups of alpha+- thalassaemia (n=533; homozygous, n=49; heterozygous, n=126; normal genotype, n=358). The differences within age group >60 and ≤ 60 months was significant, p<0.001. The differences between the age group >60 and ≤ 60 months for homozygous and heterozygous alpha+-thalassaemia were not significant (p=0.8281, p=0.7412)

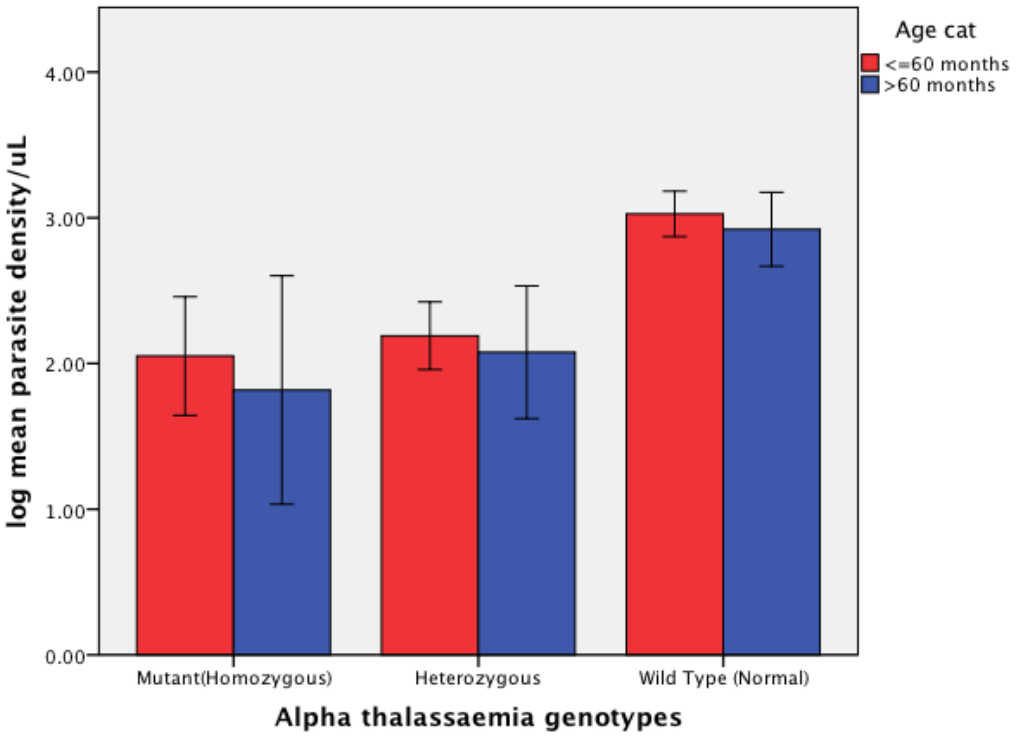

Figure 7.2:The density of malaria parasite in the various age groups of alpha+-thalassaemia.(n=533); homozygous alpha+thalassaemia-(n=49);heterozygous alpha+thalassaemia-(n=126); normal genotype-(n=358). p=0.207

Table 7.2 *Plasmodium falciparum* malaria in terms of alpha+-thalassaemia genotypes among children of ≤60 months and >60 months age group

	Mean (±SD)			
	-α/-α (n=49)	**-α/αα (n=126)**	**αα/αα (PID) (n=358)**	**P-value**
		GMPD/µL		
>60 months	3545(1.01)	10034(0.88)	4171(1.05)	<0.001
≤60 months	7980(1.02)	9015(1.00)	51794(1.11)	<0.001

Analysis of variance (ANOVA) assessed the differences in the mean values GMPD-geometric mean parasite density; α/αα, heterozygous alpha +-thalassaemia; -α/-α, homozygous alpha+-thalassaemia; αα/αα, normal or wild type alpha +- thalassaemia; Data shown represent mean and SD

7.7 Discussion

We have discussed that alpha^{+}-thalassaemias were protected from the severe manifestation of *Plasmodium falciparum*, and that the GMPD were lower amongst the homozygous and heterozygous alpha^{+} thalassaemia compared to the normal genotype (PID-presumed iron deficiency). However, researchers have agued whether this protective effect is directly linked to the alpha^{+-}-thalassaemia genotype or that the protection wanes once the patient grows out of the high-risk zone of 60 months. We grouped our subjects into children ≤60 months and those >60 months. Even though the age-group ≤60 months recorded a higher proportion of severe malaria compared to age group >60 months, the difference between the two age groups, was not significantly differently (p=0.8281 and p=0.7412) contrary to observation by Mockenhaupt *et al*., (1999a, 2004a) who reported that the protective effect of heterozygous alpha+- thalassaemia from severe *Plasmodium falciparum* parasitaemia was strongest in youngest children during which time that malaria-associated mortality is highest. In order words older children might be more susceptible to *Plasmodium falciparum* and therefore have less protection. However our observation supports findings by Allen *et al.,* (1997) that greater frequency of malaria were seen in alpha thalassaemia children with a median age of <3.6 years and Veenemans *et al*., (2008) who reported more protective immunity in older children with alpha+-thalassaemia.

The higher proportion of severe malaria in the age-group ≤60 months may have provided immunity in the older age hence the degree of parasitaemia between the age groups was also not significant (p=0.207, Figure 7.2); the lower GMPD in the homozygous alpha^{+}-thalassaemia notwithstanding. The proportion of children with severe *Plasmodium falciparum* malaria in both heterozygous and homozygous alpha^{+}-thalassaemia was significantly different compared to normal genotype within the age groups (p<0.001, Figure 7.1). This finding suggests that children irrespective of age group were protected from the severe manifestation of *Plasmodium falciparum* parasitaemia.

The number of alpha^{+}-thalassaemias who had severe *Plasmodium falciparum* were very few and this might have affected the results. This shortfall was evident amongst children >60 and who were homozygous alpha^{+}thalassaemia. The prevalence of the homozygous alpha^{+}-thalassaemia in Ghana is 2-3% hence the shortfall. We observed that this age–specific risk reduction involving *Plasmodium falciparum* and infected alpha^{+}-thalassaemic red blood cells might be an interaction between maternal immunity and the innate mechanism of alpha^{+}-thalassaemia. The age group that children acquire immunity as observed by Aidoo *et al.,* (2002) is 2-16 months. In other words increased susceptibility to malaria in infancy might result in improved immunity to subsequent severe parasitaemia by enhanced antigen expression on *Plasmodium falciparum*–infected alpha+-thalassaemic erythrocytes (William *et al.,* 1996).

The fact that malaria-associated mortality is strongest in younger children less than 60 months suggests that innate resistance might probably act before specific immunity develops.

This study found protection from *Plasmodium falciparum* in both heterozygous and homozygous alpha^{+}-thalassaemia who were either less than or more than 60 months, an observation that contrast findings by Enevold *et al.,* (2008), who investigated the risk of uncomplicated malaria in alpha^{+}-thalassaemia children and observed that the protective effect was prominent in both heterozygous and homozygous who were older than 60 month.

We are however of the opinion that the lower proportion of severe *Plasmodium falciparum* cases recorded in the age group >60 months might suggest an enhanced protection from severe malaria in both the homozygous and heterozygous alpha^{+} -thalassaemia in this age group.

7.8: Summary, limitations and further works

We observed that the proportion of *Plasmodium falciparum* parasitaemia amongst children >60 months were fewer compared to children ≤60 months. Similarly parasite count and GMPD were lower in children >60 months compared to children ≤60 months.

Statistically there was no significant difference between the two age groups; the implication is that the protection conferred by alpha+ thalassaemia on *Plasmodium falciparum* parasitaemia might not wane with age. Future study might have to consider increasing the sample size in order to enrol more homozygous alpha+ thalassaemia, which has been the limitation to these studies

Chapter 8

8.0: Final discussion

The basic proposition of Haldane's hypothesis that alpha^{+}-thalassemia is pronounced in malarious regions is because it accords carriers a level of protection against death from the severe manifestation of *Plasmodium falciparum* parasitaemia by assuaging the clinical course of the disease. (Hendricks 2003).

Evidence suggests that alpha^{+}-thalassaemia ameliorates the severity of malaria, but does not reduce initial infection rates. However, current studies have not been able to provide plausible mechanisms for the protection of alpha^{+}-thalassaemia against the severe manifestation of *Plasmodium falciparum* (Pasvol, 2006, Harteveld and Higgs, 2010).

The prevalence of *Plasmodium falciparum* malaria as provided in this study was 54.1% among children of the Kumasi metropolitan area. This is in contrast to previous observations by Asante, (2003) and Ronald *et al*., (2006) who found respectively a 13.65% and 25.30% prevalence of *Plasmodium falciparum* malaria among children in the Kumasi metropolitan area. Our finding therefore suggests that malaria is hyperendemic in Kumasi, an urban population inspite of governmental interventions against mortality due to malaria.

The prevalence of $-\alpha^{3.7}$ type of alpha+-thalassaemia was 29.3%. This compared favourably with the 20% to 30% prevalence reported by Borges *et al*., (2001) for the West African sub-region and 26%- 33% prevalence reported by Mockenhaupt *et al*., 2001; Mockenhaupt *et al*., 2004a and May *et al*., 2007). Alpha thalassaemia does not exclude the presence of iron deficiency, and however, the high prevalence of alpha^{+}-thalassaemia does suggests that alpha^{+}-thalassaemia is a key contributor to microcytosis and therefore informs the need to appropriately discriminate iron deficiency from alpha^{+}-thalassaemia to avoid the unnecessary administration of iron therapy. The high prevalence of alpha^{+}-thalassaemia might be the as a result of the protection of these children from the severe manifestation of *Plasmodium falciparum* parasitaemia (Hendricks 2003).

In the normal genotype, heterozygous and homozygous alpha^{+}-thalassaemia groups we observed a connection between a progressive decrease in MCV (increased microcytosis) and decrease in parasite density suggesting microcytosis might be associated with a reduction in parasite burden in alpha^{+}-thalassaemia (Fowkes *et al*., 2008). However, we found that both alpha+-thalassaemia genotypes were less susceptible to the severe manifestation of malaria as expressed by the low prevalence of parasitaemia in microcytic patients with an alpha+-thalassaemia genotype (-α/αα 11.9% and -α/-α 16.3%) compared to either normocytic patients or microcytic patients with a normal genotype (32.9% and 53.6% respectively) ($p<0.03$).

Similarly, geometric mean parasite density (GMPD) values were lower in the presence of an alpha-thalassaemia genotype (-α/αα GMPD 9015, n=126 and -α/-α GMPD 6852, n=49) compared to normal genotype (αα/αα GMPD 51794, n=358) ($p<0.001$). These findings whilst indicating that the severity of *Plasmodium falciparum* parasitaemia was significantly lower in both homozygous and heterozygous alpha+-thalassaemia genotypes compared to microcytic children with normal genotype also suggests the protection from severe malaria might be due to the alpha+-thalassaemia state.

The protective effect was observed in both the heterozygous and homozygous alpha+-thalassaemia but was more evident with homozygous genotype in contrast to findings by Mockenhaupt *et al.,* (2004c), who observed protective effect from *Plasmodium falciparum* malaria in only to the heterozygous form of alpha+-thalassemia. Their inability to observe protection in the homozygous alpha+-thalassemia was attributable to the small number of the genotype in the study population, statistical consideration and differences in the design and conduct of these studies. Our finding is however, consistent with suggestions by Allen *et al.,* (1997) and Williams *et al.,* (2005b) that the protective effect is visible in both genotypes, but most evident in homozygotes.

We observed reduced parasite density in alpha+-thalassaemia children, contrary to findings (Mockenhaupt *et al.,* (2004a) and Williams *et al.,* (2006) who indicated that the protection by alpha+-thalassaemia did not lower parasite densities.

Our finding however is in support of previous suggestion that red blood cells of more severe alpha+thalassaemia genotypes are less hospitable to parasite growth and multiplication (Ifediba *et al*., 1985).

The scarcity of haemoglobin has been suggested to provide a non-conducive nutrient-deficient environment for *Plasmodium falciparum* growth (Nyakeriga *et al*., 2004b). Relative to children with normal genotype haemoglobin concentrations were not significantly different suggesting that alpha+-thalassaemia might limit the decline in haemoglobin concentration in these children. This observation compares to suggestions by Veenemans *et al*., (2008) that alpha+ thalassaemia might limit the decline in haemoglobin that is not only confined to severe malaria cases but even so the protective effect might be evident when the *Plasmodium falciparum* infection is accompanied by inflammation. Both heterozygous and homozygous alpha+-thalassaemia have been implicated in preventing the decline in haemoglobin concentration in children with clinical malaria (Wambua *et al*., 2006).

In this study however, only children heterozygous for alpha+-thalassaemia had higher mean haemoglobin concentration than did children with normal genotype. This not withstanding we suggest that the protection conferred by alpha+-thalassaemia might prevent the decline in haemoglobin concentration in the severe forms of *Plasmodium falciparum* malaria.

Our data did not observe any significant correlation between hypochromasia and *Plasmodium falciparum* parasitaemia. The GMPD in the hypochromic however differed significantly from the normochromic group ($p<0.001$), recording an increase in the density of parasite compared to the normocytic group. This suggests that hypochromic alpha+ thalassaemia were not biologically advantageous in terms of protection from parasitaemia over their normochromic counterparts and did not prevent infection.

In addition we observed a progressive decrease in MCH compared to GMPD in the order of homozygous <heterozygous <normal alpha^{+}-thalassaemias, a trend that was observed among the microcytic children with alpha^{+}-thalassaemia.

This trend might explain the fact that both microcytic and hypochromic are inherited together in either iron deficiency anaemia or the thalassaemias and that microcytic hypochromic alpha^{+}-thalassaemia genotype confer protection from the severe forms of *Plasmodium falciparum* malaria.

Severe malarial anaemia (SMA) is the leading cause of mortality in malaria-affected children under the age of five years (WHO 2000).

In the opinion of May *et al.*, (2005), protection against malaria mortality among Ghanaian children who were alpha+-thalassaemic was specific and driven by protection from severe anaemia, the most prevalent malaria complication.

Suggestions were that the protection from SMA are more pronounced in homozygous alpha^{+}-thalassaemia (Wambua *et al*., 2006, William *et al*., 2005, Mockenhaupt *et al*., 2004, May *et al*., 2007); that the mechanism for this protection against SMA might be an increased in erythrocyte count and microcytosis thus maintaining the Hb concentration above 5.0g/dL (Fowkes *et al*., 2008). Contrary to these findings we observed in this study an increased susceptibility to *Plasmodium falciparum* malaria and parasitaemia among both heterozygous and homozygous alpha^{+}-thalassaemia whose haemoglobin concentration were ≤5.0g/dL compared to those with haemoglobin concentration of > 5.0g/dL. Our finding suggests that SMA might be implicated in increased GMPD among heterozygous and homozygous alpha^{+}-thalassemia.

The protective effect from the severe manifestation of *Plasmodium falciparum* by alpha^{+}-thalassaemias have been discussed and that the GMPD were lower among the homozygous and heterozygous alpha^{+} thalassaemia compared to the normal genotype but whether this protective effect is directly linked to the alpha^{+-}-thalassaemia genotype or that the protection wanes once the patient grows out of the high-risk zone of 60 months was investigated. Within the age groups of children >60 months and children ≤60 months, the proportion of severe *Plasmodium falciparum* and GMPD were significantly different between the homozygous and heterozygous alpha^{+}-thalassaemia compared to the normal genotype ($p<0.001$).

Between children >60 months and ≤60 months *Plasmodium falciparum* densities for the homozygous, heterozygous and normal alpha^{+} thalassaemia genotypes were not statistically significant (p=0.207). Our findings suggest that alpha^{+} thalassaemia children irrespective of age group were protected from the severe manifestation of *Plasmodium falciparum* parasitaemia and by implication the protection conferred by alpha+ thalassaemia against severe *Plasmodium falciparum* parasitaemia might not wane with age.

The frequency of alpha+- thalassaemia in the study population was 29.3% and the fact that iron deficiency (ID) occurs in alpha+-thalassaemia might require that future study discriminate ID from alpha+-thalassaemia before investigating the protective effect from *Plasmodium falciparum* parasitaemia. The epistasis relationship between notable haemoglobinopathies like Hb AS and HbAC and alpha+-thalassaemia have been suggested to erode the protection conferred by alpha+-thalassaemia against *Plasmodium falciparum* parasitaemia. Therefore it might be prudent to investigate this interaction as it could provide important information why malaria mortality is high in this population. Alpha+-thalassaemia has been suggested to protected decline in haemoglobin concentration however severe anaemia as observed in this study resulted in increased parasitaemia Further works might have to discriminate other causes of hypochromic and microcytic anaemia like worm infestation and examine why severely anaemic children were not protected from *Plasmodium falciparum* parasitaemia. Finally increasing the sample size and the age of subjects could enable the enrolment of more homozygous alpha+ thalassaemia, which has been the limitation to this study

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