RELATION BETWEEN ALPHA^{+}-THALASSEMIA AND GLUTATHIONE-*S*-TRANSFERASES POLYMORPHISMS IN CHILDREN WITH SEVERE MALARIA IN TANZANIA

BY

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ABSTRACT

Malaria remains a major public health problem in Sub-Saharan Africa. In Tanzania, malaria is believed to cause about 30% annual episodes and 15% death compared to other diseases. Alpha^{-}-thalassemia and common *glutathione-S-transferase* (*GST*) genotypic polymorphisms have been reported independently to confer protection against severe malaria, but the molecular basis for their individual protection is largely unknown. The relationship between genotypic polymorphisms of alpha^{+}-thalassemia and *GST* in children with severe malaria was determined in a cross sectional-retrospective study using 148 children aged between 1 and 15 years. The study was conducted at Mnyuzi village in Korogwe district, Northeastern of Tanzania. *Glutathione-S-transferase-pi1* (*GSTP*1)-polymorphism was observed to have almost three fold risk (OR = 2.9; 95% CI =1.3- 6.1; P = 0.006) of developing severe malaria in children compared to mild malaria. In the presence of *GSTP*1, decrease in protective effect of alpha^{+}-thalassemia polymorphisms (homozygotes and heterozygotes) against severe malaria was observed from OR = 0.81 (95% CI = 0.5-1.5; P = 0.5) to OR = 0.78 (95% CI = 0.4-1.5; P = 0.44). This study concludes that *GSTP*1 polymorphism increases malaria severity. Also there is slight inverse relationship between *GSTP*1 polymorphisms and alpha^{-}-thalassemia to children with severe malaria.

DECLARATION

I, Fredy Saguti. do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and it has neither been submitted nor being concurrently submitted in any other institution.

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The above declaration is confirmed

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(Supervisor)

18 · 11 · 2011
Date

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DEDICATION

I dedicate this dissertation to my wife Joanna Saguti.

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LIST OF ABBREVIATIONS AND SYMBOLS

μλ	Microlitre
CI	Confidence interval
DNA	Deoxyribonucleic acid
G6PD	Glucose-6-phosphate dehydrogenase
GSH	Glutathione
GST	Glutathione-*S*-transferase
GSTA	Glutathione-*S*-transferase-alpha (α)
GSTM	Glutathione-*S*-transferase-mu (μ)
GSTP	Glutathione-*S*-transferase- pi (π)
GSTT	Glutathione-*S*-transferase- theta (θ)
*GSTP*1	Glutathione-*S*-transferase-pi 1
*GSTM*1	Glutathione-*S*-transferase-mu 1
*GSTT*1	Glutathione-S-transferase-theta 1
Hb	Hemoglobin
IQR	Interquantile range
MCV	Mean corpuscular volume
min	Minutes
ml	Millilitre
Mm	Millimeter
mM	miliMole
n	Sub-total number

N	Total number
OR	Odds ratio
O^{-}_{2}	Superoxide radical
PCR	Polymerase chain reaction
R1	Reverse primer 1
R2	Reverse primer 2
RBCs	Red blood cells
RNA	Ribonucleic acid
ROS	Reactive oxygen species

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information and Justifications

Malaria morbidity is estimated to be 300-500 million cases worldwide and about one million deaths each year (Snow *et al.*, 2005). Malaria is a major public health problem especially in Sub-Saharan Africa (Kilama, 2003; Kilama 2006). In Tanzania, malaria is estimated to cause about 30% annual malarial episodes and 15% death (Ministry of Health, 2001). Malaria is caused by protozoan parasites of the genus Plasmodium. The predominant human malaria species is *Plasmodium falciparum* accounting for more than 90% of all infections, the rest being *Plasmodium malariae, Plasmodium vivax* and *Plasmodium ovale. Plasmodium falciparum* causes the majority of severe and complicated malaria cases and is the leading cause of death in children in Africa (Suh *et al.*, 2004; Snow *et al.*, 2005).

Malaria infection results in increased reactive oxygen species (ROS) as well as reduction of erythrocytic antioxidants (Pagola *et al.*, 2000). The imbalance between excess of ROS and reduction of erythrocytes antioxidants has been defined as oxidative stress (Sohail *et al.* 2010). In malaria, ROS increase following intracellular parasite's metabolism on hemoglobin and release of highly reactive heme. The heme can react with molecular oxygen to form hemin and superoxide radical (O_2^-), a highly reactive oxygen species (Muller, 2004). In children with malaria, blood plasma and erythrocytic lipid peroxidation products were increased while erythrocytic antioxidants such as reduced glutathione (GSH) were reported to be

lower in patients when compared with control (Becker *et al.*, 2004). The effect of oxidative stress in malaria is largely unclear. Some study has demonstrated a protective role by indicating that malaria parasites are highly susceptible to alterations in redox equilibrium (Mannervik and Danielson, 1988). Other suggested that oxidative stress contributes to disease manifestation including sequestration, cerebral pathology, anemia, respiratory distress and placental malaria (Dockrell *et al.*, 1986; Mannervik and Danielson, 1988).

Oxidative stress is regulated by a number of mechanisms including the *GST*. *Glutathione-S-transferase* is a family of enzymes which catalyze the metabolism of electrophilic compounds of endogenous and exogenous origin by conjugating them with GSH. In human, *GST* consists of family of proteins; classified into four major classes namely *GST*-alpha (*GSTA*), *GST*-theta (*GSTT*), *GST*-Mu (*GSTM*) and *GST*-Pi (*GSTP*). Recently, studies have shown strong association between impaired or deficiency of *GSTs* activity and pathogenesis of malaria (Kavishe *et al.*, 2006; Kavishe *et al.*, 2009; Sohail *et al.*, 2010).

It has been proposed that, high prevalence of hemoglobinopathies in malaria endemic areas reflected a balance polymorphism in which the deleterious consequences of homozygosite are offset by the protection from severe *Plasmodium falciparum* malaria conferred by the heterozygous state (Haldane, 2004). The hypothesis has been tested to be true in a variety of erythrocytic inherited genetic traits including alpha^{+}-thalassemia (Veenemans *et al.*, 2008; Hedrick, 2011).

Alpha^{+}-thalassemia is an inherited genetic disorder of hemoglobin synthesis: a deficiency in biosynthesis of one or both copies of α-globin gene on chromosome 16. Trans-deletion of alpha alleles on different chromosomes is known as α-homozygotes (-α/-α) or (--/αα), while deletion of the single alpha allele is α-heterozygotes (-α/αα) (Weatherall, 1980). Alpha^{+}-thalassemia prevalence has consistently been reported to be both high (Flint *et al.*, 1986; Fodde *et al.*, 1988) as well as conferring partial protection to severe malaria (Mockenhaupt *et al.*, 2004; Williams *et al.*, 2005; Veenemans *et al.*, 2008). However, the mechanism of its protection remains largely unknown. In Tanzania where malaria is endemic, the prevalence of alpha^{+}-thalassemia has been reported to reach up to 55% (Enevold *et al.*, 2007).

1.2 Problem Statement and Justification

Examination of alpha+-thalassemia and *GST* polymorphisms and their interaction with severe malaria has not been determined before. Recently, Kavishe *et al.* (2009) determined the prevalence of common genotypes of *GST* polymorphisms in mild *versus* severe malaria children in Tanzania. The study showed an association between *GSTP1* I105V and severe malaria anemia (Kavishe *et al.*, 2009). In attempt to further understand the relationship between *GST* polymorphisms and inherited alpha^{+}-thalassemia in children with severe malaria, an investigation on the relationship between common *GST* and alpha^{+}-thalassemia polymorphisms in terms of disease susceptibility and protection was carried out in this study. The research was conducted to detect alpha^{+}-thalassemia genotypic polymorphisms from the same

DNA samples from children aged between 1 to 15 years old in malaria endemic area of Mnyuzi, Korogwe district, north eastern of Tanzania.

1.3 Objective of the Study

1.3.1 Overall objective

Broadly the objective of this study was to determine the relationship between genotypic polymorphisms of alpha-$^{+}$thalassemia and *GST* in children with severe malaria.

The specific objectives of the study were:

1.3.2 Specific objectives

1. To determine the prevalence of alpha^{+}-thalassemia polymorphism in children with mild *versus* severe malaria
2. To determine the risk of developing severe falciparum malaria in the presence of *GST* polymorphism
3. To investigate relationship between alpha^{+}-thalassemia and *GST* genotypic polymorphisms in children with severe malaria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Thalassemia

Thalassemia originated from the Greek words thalassa (sea) and haima (blood), a group of inherited autosomal recessive hematologic disorders. Thalassemia causes hemolytic anaemia due to either decrease or absence of a globin chain. Theoretically, there are two types of thalassemia which are alpha^{+}- thalassemia and beta thalassemia. While alpha^{+}-thalassemia occurs most in persons from Africa and Southeast Asia, beta thalassemia is more prevalent in Mediterranean as well as Southeast Asian and African regions (Muncie and Campbell, 2009).

Normally, hemoglobin appears as tetrameric protein with four polypeptide chains consisting of two alpha chains and two beta chains. The chains are held together by non-covalent bonds interaction. The heme moiety is attached in the knot of polypeptide globin formed by amino acids. This carries a molecule of oxygen with the help of ferrous ions (Fe^{++}) which has a valence corresponding to that of oxygen molecule (Perutz, 1979).

The alpha gene is duplicated, and there are two alpha globin genes per haploid genome. Alpha^{+}-thalassemia is the result of deficiency or absence of production of alpha globin chains substitute with beta globin chains. The deficiency of production of alpha globin chains is due to the deletion of one to four of alpha globin genes in chromosome 16. Trans deletion of alpha alleles on different chromosomes results

into homozygotes (--α/--α). while deletion of a single alpha allele gives heterozygotes (--α/αα) (Bernini and Harteveld, 1998; Harteveld and Higgs, 2010). Also alpha+-thalassemia is usually inherited in a Mendelian recessive fashion (Steensma *et al.*, 2004; Muncie and Campbell, 2009)

The acceptable classification of alpha thalassemia is α^0 when there is normal α globin produced by the gene, and α^+ when there is reduced globin production. Alpha$^+$-thalassemia is characterized by reduction in erythrocytic volume and size. This is in turn associated with decrease in mean corpuscular volume (MCV), a process known as microcytosis. There is an overall reduction in erythrocytic hemoglobin (Hb) concentrations, which are considered to cause anaemia. These changes are more pronounced in homozygous as compared to heterozygous individuals who phenotypically are considered to be normal (Danquah and Mockenhaupt, 2008).

2.2 Alpha$^+$-Thalassemia

Alpha$^+$-thalassemia has been consistently shown to confer protection against uncomplicated and severe malaria. This protection is mostly pronounced in homozygotes than heterozygote patients (Mockenhaupt *et al.*, 2004; Williams *et al.*, 2005). Although the mechanisms for protection are largely unknown, alpha$^+$-thalassemia is claimed to prevent disease progression through mechanisms other than limiting parasite replication as well as to severe manifestation of disease (Pasvol, 2006; Williams, 2006).

The protective advantages conferred by alpha+-thalassemia against malaria have been consistently reported throughout in malaria endemic areas in Africa. In Nigeria, malaria associated anaemia was shown to be less pronounced in non-hospitalized children with heterozygote alpha^{+}-thalassemia than children with normal genotype and virtually absent in homozygotes (Mockenhaupt *et al.*, 1999). Similarly, a study in predominantly asymptomatic pregnant women in Ghana revealed less effect on falciparum malaria infection in hemoglobin concentration in women with α^{+}-thalassemia than their counterparts (Mockenhaupt *et al.*, 2000). In Tanzania and Kenya, α^{+}-thalassemia was shown to protect the decline in hemoglobin concentration associated with mild or asymptomatic falciparum malaria infection. The protection was more observed in homozygotes than in heterozygotes and was observed when infection was accompanied by inflammation (Veenemans *et al.*, 2008).

2.3 Glutathione-*S*-Transferases

The glutathione transferases (EC 2.5.1.18) have historically also been called glutathione-*S*-transferases. It is this latter name that gave rise to the widely used abbreviation, *Glutathione-S-transferase*. It represents a large family of conjugating enzymes containing at least seven isozymes (Hayes *et al.*, 2005).

Glutathione-S-transferases are widely distributed in three major cell compartments namely, mitochondria, cytosol and microsomes. The mitochondrial *GST* consists of the kappa class. The microsomal *GST* also referred to as membrane-associated proteins eicosanoids and glutathione (MAPEG) and is unrelated to the soluble *GST*

(Jakobsson *et al.*, 1999). They are membrane-bound *GSTs* which are also involved in cellular signaling as well as detoxification of ROS (Frova, 2006). Cytosolic GST comprise a family of proteins classified into five major groups. *GST*-Alpha, Mu, Theta, Pi, and Zeta. The most characterized among the families are cytosolic *GST*-alpha, Mu and Pi (Salinas and Wong, 1999; Hayes *et al.*, 2005).

Glutathione-*S*-transferases play an important role in the metabolism of xenobiotics such as carcinogens, environment pollutants and antitumor agents (Hayes *et al.*, 2005). *GSTs* is also important is detoxifying endogenous alpha, beta-unsaturated aldehydes, quinines, epoxides, and hydro-peroxides. All these are done by virtue of their conjugation reactions with glutathione (GSH). Glutathione-S-transferases are also intimately involved in the biosynthesis of leukotrienes, prostaglandins, testosterone and progesterone (Boyer and Olsen, 1991; Hayes *et al.*, 2005). In case the potentially toxic electrophiles are not conjugated to GSH, they might be free to combine covalently with DNA, RNA, or cell protein and could thus lead to serious cell damage (Mannervik and Danielson, 1988; Salinas and Wong, 1999).

2.4 Glutathione-*S*-Transferases Polymorphisms and Malaria

Glutathione-*S*-transferases have been among enzymes that modulate inflammatory processes. Therefore, polymorphisms in *GSTs* resulting into impaired or deficiency in enzymatic activity have received attention in various disease epidemiological studies including leukemia and malaria (Ye and Song, 2005; Kavishe *et al.*, 2006; Sohail *et al.*, 2010).

Glutathione-*S*-transferases polymorphisms are suggested to increase oxidative stress in malaria infections (Kavishe *et al.*, 2006). The enzymatic deficiency tends to decrease glutathione (GSH) concentration and increase disease manifestation. When toxic reactive oxygen species are not conjugated or inactivated with antioxidant such as glutathione, they may lead to red blood cells damage and disease manifestation (Becker *et al.*, 2004).

2.5 GST Polymorphisms and Alpha^{+}-Thalassemia

Alpha^{+}-thalassemia is considered to be among the world's most common single gene disorders (Higgs *et al.*, 1989). Although it has been reported consistently that alpha^{+}-thalassemia confer protections, little is known about its malaria protective effects. Attempts have been made to find it's interaction with other modulating factors for malaria such as sickle cell anaemia and glucose-6-phosphate dehydrogenase (G6PD) deficiency.

In Tanzania, the risk of suffering from febrile, uncomplicated malaria between individuals carrying three common red blood cell (RBC) polymorphisms (alpha^{+}-thalassemia, *G6PD* deficiency and sickle cell trait) and control were studied (Enevold *et al.*, 2008). In this study alpha^{+}-thalassemia was associated with a reduced risk of uncomplicated malaria episodes especially to children older than 5 years of age.

Recently, *GST* polymorphisms have attracted attention in malaria. In a study conducted in Cameroon, *GSTM1* was also shown to be associated with complicated

malaria when compared with uncomplicated malaria (Kavishe *et al.*, 2006). Similarly in the study conducted in Tanzania *GSTP*1 I105V was shown to be significantly associated with severe malaria anaemia (Kavishe *et al.*, 2009). In India, malaria pathogenesis was shown to be associated with polymorphism of *GSTP*1 (Sohail *et al.*, 2010). It is unknown whether *GST* polymorphisms have any interactions with other common genetic disorders such as alpha^{-}-thalassemia to children with severe malaria or not. Therefore, the current study has tried to investigate whether there is any relationship between *GST* and alpha^{+}-thalassemia polymorphism in falciparum malaria infected individuals.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area and Population Characteristics

The study was conducted using blood samples collected from children aged 1 to 15 years residing at Mnyuzi, a rural village in the Tanga Region, north eastern of Tanzania. Samples were collected in a period between Julys through September 2006. Malaria transmission intensity was high with an estimated entomological inoculation rate (EIR) of 91 infectious bites per person per year (Lusingu *et al.*, 2004). The rain pattern is bimodal, with a long rainy season between March and June, and short rainy season between October and December.

Participants were recruited at Mnyuzi Health Centre. Total of 148 children between 1–15 years of age with a body temperature above 37.5°C or a history of fever within the last 48 hours were enrolled after a written informed consent from their parents/guardians. Furthermore children with *Plasmodium falciparum* mono-infection at a density between 500 and 100,000 parasites/μL were eligible for recruitment. Children with hemoglobin (Hb) concentration below 8 g/dL, hyper-parasitaemia (≥ 250,000 parasites/μL) and metabolic acidosis manifested by respiratory distress as described by Marsh *et al.*, (1995) were considered as severe cases. Also cerebral malaria presented as coma score ≤ 2 (Blantyre coma scale) or impaired consciousness with Blantyre score < 3 and prostration or extreme weakness were recruited as severe malaria group.

For severe cases treatment was initiated with quinine, according to Tanzanian National Guidelines and referred to the nearby district hospital in Korogwe in case the study physician considered this appropriate. There was no active follow-up of the outcome of severe malaria cases after the appropriate treatment was installed.

3.2 DNA Extraction

The DNA samples were extracted from filter paper blood blots of children infected with *Plasmodium falciparum* malaria. Nucleospin Tissue Kit (Macherey-Nagel) was used for DNA extraction as described in the manufacturer's protocol and stored at -20°C.

3.3 Identification of Alpha^{+}-Thalassemia Genotypic Polymorphisms

All chemicals for PCR including primers used in genotyping of alpha^{+}-thalassemia were from New England BioLabs. Multiplex Polymerase Chain Reaction (PCR) was used. PCR products were separated in 0.75% agarose gel. The PCR protocol was adopted from Liu *et al.*, (2000). Oligonucleotide primers 5'-AAGTCCACCCCTTCCTTCCTCACC-3' as sense and 5'-ATGAGAGAAATGTTCTGGCACCTGCACTTG-3' (R1) as antisense were used to amplify the wild-type alpha-thalassemia (-$\alpha^{3.7}$). Also Oligonucleotide primers for -$\alpha^{3.7}$ gene 5'-AAGTCCACCCCTTCCTTCCTCACC-3' as a sense primer and 5' TCCATCCCCTCCTCCCGCCCCTGCCTTTTC-3' (R2) an antisense were used to amplify homozygote alpha^{+}-thalassemia named -$\alpha^{3.7}$. The PCR reaction mixture constituted 25µl which included: 100% of DMSO, 3.74µl of betaine (5M), 100 - 150ng genomic DNA, 1.5µl of 25mM of each dNTPs, 0.1µl of 20µM of each primer,

0.75ul of 50mM $MgCl_2$, 0.25µl of 5 units of Taq polymerase and 2.5µl of 10xTaq polymerase PCR buffer. The RNase free water was used as control instead of genomic DNA.

The cycling conditions were: initial denaturation at 95°C for 16 min, followed by 35 cycles of 60sec denaturation at 95°C, 60 sec for primer annealing at 62°C. 150sec extension at 72°C and 10min final extension at 72°C. The identification of alpha$^+$-thalassemia gene polymorphisms was carried out as described by Liu *et al.* (2000).

3.4 Gel Electrophoresis

The PCR amplicons were allowed to run in the 0.75% agarose gel at 120V and 60Am for 30min. After gel electrophoresis, a picture was taken for every plate and scores were recorded on excel sheet for statistical analysis. An individual was recorded as having a wild type of alpha+-thalassemia gene if only one upper band of 2200bp was seen and homozygotes of alpha+-thalassemia if only one low band of 1900bp was seen. For heterozygotes, an individual was recorded only if two bands of both 2200bp and 1900bp were seen.

3.5 Statistical Analysis

Data were analyzed using Stata/MP (version 10.0). The proportion of both alpha$^+$-thalassemia and *GSTs* genotypic polymorphisms were calculated and compared in mild *versus* severe malaria individuals. Pearson's chi-square test was used to analyze the strength of association between genotypic polymorphisms in mild *versus* severe malaria. Logistic regression models analyses were done to describe the magnitude of

association between either alpha+ thalassemia (for the *GSTs* polymorphisms analysis) or *GSTs* polymorphisms (for the alpha+-thalassemia analysis) as independent variables, and severe malaria together with age as the dependent variables.

3.6 Ethical Clearance

The ethical clearance for the study was obtained from the Tanzanian National Institute for Medical Research (NIMR/HQ/R.8a Vol. XIII/446) and Kilimanjaro Christian Medical Centre (KCMC 2006#28). Informed consents were obtained from the parents or guardians of the children.

CHAPTER FOUR

4.0 RESULTS

4.1 General Characteristics

The general characteristics of the study population are shown in Table1. Fifty percent (80/148) of the children were males and 45.9% (68/148) were females. Out of 148 children infected with malaria, 95 had mild malaria while 53 had severe malaria. In terms of gender, 53.7% of males had mild malaria when compared with females with mild malaria. Of severe malaria subjects, about 54.7% were males and 43.3% were females. Median age in the severe malaria group was 8.0 years with interquantile range (IQR) of 5.0-11.0. while in mild malaria median age was 5.0 years and IQR 3.0-9.0. Hemoglobin concentrations were 4.3g/dL and 10.6g/dL in severe and mild malaria respectively. The mean temperature for severe malaria was 38.45°C and 37.3°C in mild malaria.

All 53 children with severe malaria were found to have severe anaemia without hyperparasitemia. Furthermore 5.6%, 7.5% and 3.7% of these children had respiratory distress, prostration and reduced consciousness respectively.

4.2 Prevalence of *GST* Gene Polymorphisms

Glutathione-S-transferase-mu1 genotype had high prevalence in severe malaria (46.7%) than in mild malaria (25.3%), although not statistically significant (P = 0.069) (Table 2). *Glutathione-S-transferase-theta1* genotype in mild group had prevalence which was comparable to that of the severe malaria group.

Table 1: General characteristics of mild *versus* severe malaria in children with *falciparum* malaria

	Mild	Severe
N	95	53
Gender, % male (n/*N*)	53.1 (51/95)	54.7(29/53)
Age, median (IQR)(years)	5.0 (3.0–9.0)	8.0 (5.0–11.0)
Hemoglobin concentration, median g/dL (IQR)	10.6 (9.7–11.9)	4.3(3.8-4.8)
Temperature, median (IQR)	37.3 (36.9–38.0)	38.4 (37.8–39.0)
Clinical Signs		
Severe anemia, % (n/ *N*)	-	100.0 (53/53)
Hyperparasitemia, % (n/ *N*)	-	0.0 (0/53)
Respiratory distress, % (n/ *N*)	-	5.6 (3/53)
Reduced consciousness, % (n/ *N*)	-	3.7 (2/53)
Prostration, % (n/ *N*)	-	7.5 (4/53)

Table 2: Prevalence genotypic allelic of *glutathione-S-transferases* gene polymorphisms in mild and severe malaria

	Mild	Severe	χ^2	*P* value
GSTM1 -null, % (n/ *N*)	25.3 (24/95)	46.7 (21/53)	3.315	0.069
GSTT1 -null, % (n/ *N*)	47.7 (47/95)	52.8 (28/53)	0.406	0.524
GSTP1				
Wild type, % (n/ *N*)	38.95 (37/95)	15.1 (8/53)		
Heterozygous mutant , % (n/ *N*)	50.5 (48 /95)	60.4 (32 /53)		
Homozygous mutant, % (n/ *N*)	10.5 (10/95)	24.5 (13/53)		0.004

The prevalence of homozygous *GSTP1* mutants in the severe malaria group (24.5 %) was significantly higher ($P = 0.004$) than in the mild group (10.5 %) with consisted of heterozygote and homozygote. The prevalence of common *GST* gene polymorphisms in children with *falciparum* malaria in Tanzania is further illustrated in Fig. 1.

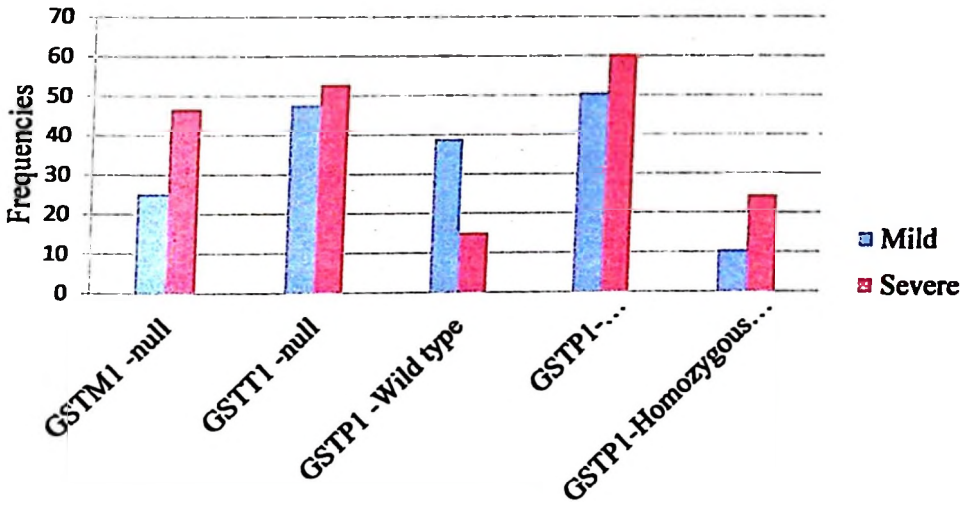

Figure 1: Frequency of *GST* genotypic polymorphisms in mild and severe malaria

4.3 Prevalence of Alpha$^+$-Thalassemia Gene Polymorphisms

PCR products were run on 1% agarose gel as shown in Fig. 2: (Lanes 1: ladder marker; 2-4: three different samples; 5: negative control; 6: homozygous; 7: heterozygous and 8: wild type). The prevalence of alpha$^+$-thalassemia was 35.8% for heterozygous and 4.7% for homozygous. When heterozygous and homozygous were combined together a carriage rate of 40.5% was obtained (Table 3).

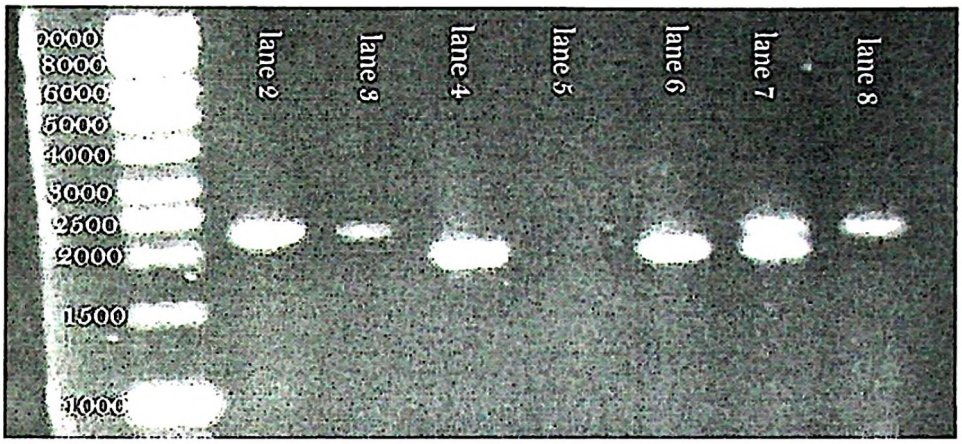

Figure 2: **DNA gel electrophoresis showing PCR products for alpha^{+}-thalassemia**

Table 3: **Frequency of alpha^{+}-thalassemia gene polymorphisms in children infected with malaria**

Alpha^{+} -thalassemia	Frequency	Percentage%
Wild type	88	59.5
Heterozygote mutant	53	35.8
Homozygote mutant	7	4.7

4.4 Alpha^{+}-Thalassemia Polymorphisms in Malaria Infected Children

The prevalence of alpha+-thalassemia genotypes in mild malaria was wild type 58.9% for wild type, 33.6% for heterozygous and 7.4% for homozygous genotype (Fig. 3). The prevalence in severe malaria was 60.4% for wild type and 39.6% for heterozygous genotype (Table 4).

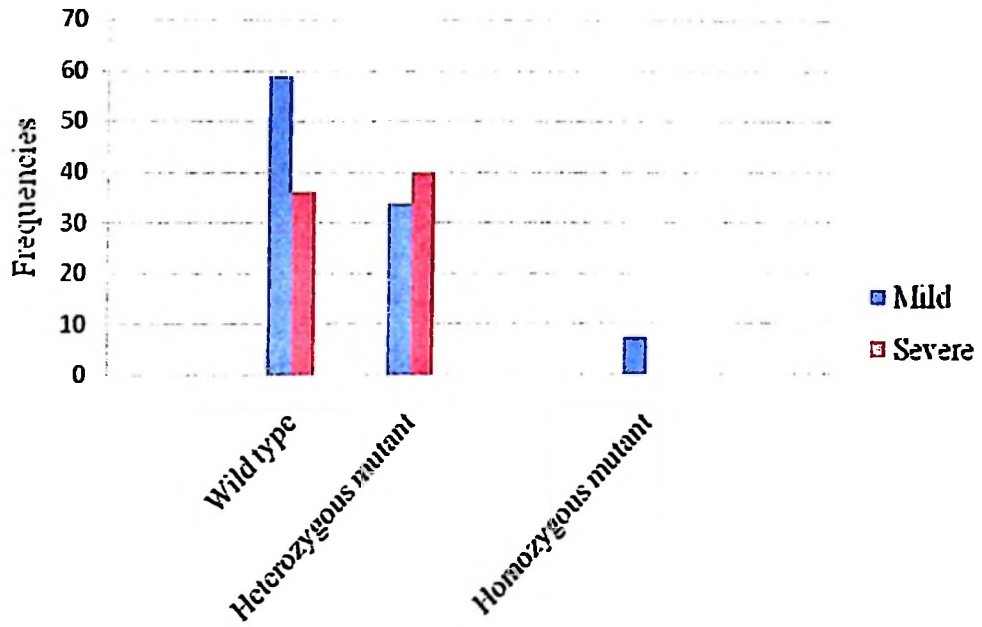

Figure 3: **Genotypic frequency of alpha+-thalassemia in mild and severe malaria in children.**

Table 4: **Genotypic proportion of alpha$^+$-thalassemia gene polymorphisms in mild *versus* severe malaria in children**

	Mild	Severe		*P value*
N	95	53	χ^2	
Alpha$^+$ -thalassemia				
Normal %(n/*N*)	58.9 (57/95)	60.3 (32/53)		
Heterozygous mutant % (n/*N*)	33.6 (32/95)	39.6 (21/53)		
Homozygous mutant %(n/*N*)	7.3 (7/95)	0 (0)	4.252	0.119
Combined wild type, %(n/*N*)	60 (57/95)	60.4 (32/53)		
Heterozygote and homozygote, %(n/*N*)	40 (38/95)	39.6 (21/53)	0.002	0.964

4.5 Interaction Between *GST* and Alpha^{+}-Thalassemia in Children with Severe Malaria

Regardless of age involved in the regression models, alpha^{+}-thalassemia traits (heterozygotes and homozygotes) was shown to (OR = 0.81; 95% CI = 0.45-1.48; *P* = 0.50) confer protection to children against severe malaria compared to mild malaria. *GSTP1* polymorphisms (heterozygous and homozygotes) had two fold risk (OR =2.58; 95% CI = 1.46-4.54; *P* = 0.001) in children to developing severe malaria compared to mild malaria. Both *GSTT*1 and *GSTM*1 mutants (nulls) showed protection to children against developing severe malaria compared to mild malaria. Further illustration using crude odds ratio of genotype polymorphisms of alpha^{+}-thalassemia and *GSTs* are shown in Table 5. When alpha^{+}-thalassemia was adjusted with common *GSTs* polymorphisms (*GSTM*1, *GSTP*1 and *GSTT*1) to detect the risk of children to develop severe malaria, the protective effect of alpha^{+}-thalassemia was slightly decreased compared to when not unadjusted. The decrease of alpha+-thalassemia protection (OR = 0.78; 95% CI = 0.43-1.45; P = 0.44) was associated with the presence of *GSTP*1 mutants (Table 6).

Table 5: The risk of children less than five years old to develop severe malaria per individual genotypic polymorphisms

Genotypes polymorphisms	Crude Odds ratio	95% CI	*P* value
Alpha+-thalassemia mutants	0.81	0.45-1.48	0.5
GSTP1 mutants	2.58	1.46-4.54	0.001*
GSTM1 null	0.53	0.26-1.09	0.084
GSTT1 null	0.8	0.41-1.57	0.52

4.6 Association between *GST* and alpha^{+}-thalassemia polymorphisms to under five year old children with severe malaria

The results show that about 36% (53) of children had severe malaria. Among these 64% (34) were aged less than 5 years old. The risks of developing severe malaria to children aged less than five years were two fold compared to children above 5 years old (OR = 2.47; 95% CI =1.23 - 4.97; P = 0.01).

Table 6: Decrease of alpha^{+}-thalassemia odds ratio when adjusted with *GSTP*1 mutants

Genotypes polymorphisms	Adjusted Odds ration	95% CI	*P* value
*GSTP*1 mutants	2.59	1.48-4.57	0.001
Alpha+-thalassemia mutants	0.78	0.43-1.45	0.44

Logistic regression models for common glutathione-*S*-transferases polymorphisms adjusted for the alpha+-thalassemia as a confounding factor to age showed almost 3 fold increased risk to develop severe malaria to children less than five years old (OR = 2.88; 95% CI =1.35 - 6.12; P =0.006) compared to mild malaria (Table 7). This effect was significantly associated with *GSTP*1 mutants rather than alpha+-thalassemia, *GSTT*1 nulls and *GSTM*1 nulls.

When alpha+-thalassemia mutants (heterozygotes and homozygotes) was adjusted only for *GSTP*1 mutants (heterozygotes and homozygotes) as cofounding to age, the protective effect of alpha+-thalassemia decreased compared to when adjusted for the rest of *GSTs* polymorphisms (OR = 0.86; 95% CI = 0.45-1.63; *P* = 0.65). However, alpha+-thalassemia was found to confer significant protection (OR = 2.9; 95% CI = 1.37-6.13; P = 0.005) to children less than five years old if they carried GSTP1 mutants (Table 8).

Table 7: Adjusted odd ratio of age, alpha^{+}-thalassemia and *GSTs* polymorphisms in severe malaria

Genotypic polymorphisms	Adjusted odds ratio	95% CI	*P* value
Age	2.88	1.35-6.12	0.006
*GSTP*1 mutants	2.79	1.54-5.06	0.001
Alpha+-thalassemia mutants	0.89	0.47-1.69	0.73
*GSTM*1 nulls	0.59	0.27-1.29	0.19
*GSTT*1 nulls	0.84	0.41-1.76	0.655

Table 8: Showing GSTP1 polymorphisms and alpha^{+}-thalassemia polymorphisms adjusted with age in severe malaria

Genotypes polymorphisms	Adjusted Odds ratio	95% CI	*P* value
Age	2.9	1.37-6.13	0.005
GSTP1 polymorphism	2.87	1.59-5.17	0.0001*
Alpha+-thalassemia polymorphisms	0.86	0.45-1.63	0.65

CHAPTER FIVE

5.0 DISCUSSION

5.1 Alpha^{+}-Thalassemia and Malaria

From this study high prevalence of heterozygotes alpha^{+}-thalassemia in both mild and severe malaria groups of children infected with *Plasmodium falciparum* malaria was observed when compared with homozygotes. Similar findings have been reported elsewhere (Enevold *et al.*, 2008). The detection of high level of heterozygous compared to homozygous alpha+-thalassemia. support a balance polymorphism hypothesis of alpha^{+}- thalassemia polymorphisms in populations exposed to endemic area with high levels of malaria transmission intensity as postulated by Haldane (2004). Alpha^{+}-thalassemia was shown to confer protection against severe malaria from infected individuals in this study. The mechanisms behind its protective effective remain largely unknown. However, alpha+-thalassemia has been claimed to prevent disease progression through mechanisms other than limiting parasite replication as well as severe manifestation of disease (Pasvol, 2006; Williams, 2006). Recently, alpha^{+}-thalassemia has been reported to use its protective effect against severe malarial anaemia by preventing the gradual decline in hemoglobin concentrations during mild *Plasmodium falciparum* infections (Veenemans *et al.*, 2008).

5.2 The Risk of Developing Severe Malaria in The Presence of GST Polymorphisms

Also from this study, *GSTP*1 polymorphisms were significantly associated with increased risk of development of severe malaria due to *Plasmodium falciparum*

compared to wild type in children aged less than 5 years old. These children had almost three fold risks to develop severe malaria. In India, *GSTP*1 polymorphism has been reported to be significantly associated with increased risk of malaria pathogenesis to carrier individuals when compared with health subjects (Sohail *et al.*, 2010). Individuals with *GSTP*1 polymorphism have been reported to have up to four-fold increased risks to malaria pathogenesis due to *Plasmodium vivax*. This might indicate that, regardless of malaria parasite species variation, *GSTP*1 polymorphisms are involved in severity of malaria. Erythrocytic antioxidant including *GSTP*1 is supposed to regulate an increase of ROS. However, as reviewed by Sohail *et al.*, (2010) the imbalance between excess ROS and antioxidants cause oxidative stress. In malaria, ROS increase after intracellular parasite's metabolism on hemoglobins and release of heme. When heme reacts with oxygen molecule, hemin and highly ROS including $O^{\cdot}_2$ are formed (Muller, 2004). Increase in oxidative stress has been associated with disease severity (Dockrell *et al.*, 1986; Mannervik and Danielson, 1988). Therefore, an increased risk of severe malaria observed from children in this study might be due to an increase in oxidative stress which is associated with an increase of *GSTP*1 polymorphisms.

5.3 Interaction Between Alpha^{+}-Thalassemia and GSTS Genotypic Polymorphisms to Children With Malaria

When alpha^{+}-thalassemia and *GSTP*1 polymorphisms were analyzed for their interaction in malaria individuals in logistic regression model, alpha^{+}-thalassemia was shown to decrease its protective effect upon existence of *GSTP*1 polymorphisms. These findings suggest the dominance effect of *GSTP*1

polymorphisms over alpha^{+}-thalassemia polymorphisms. The low frequency of alpha^{+}-thalassemia to children with severe malaria might be a reason behind the dominant of *GSTP*1 polymorphisms. although independently alpha^{+}-thalassemia still showed its protective effect against malaria. This finding indicate an inversely relationship between alpha^{+}-thalassemia and *GSTP*1 polymorphism in children with severe *Plasmodium falciparum* malaria. An inversely relationship between genetic factors which lead to disease manifestation against those which confer protection is of great importance in malaria epidemiology. In this instance, malaria severity is contributed by the dominance of the genetic factors (e.g. *GSTP*1 polymorphisms) which lead to disease severity over those which confer partial protection (alpha^{+}-thalassemia).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study has revealed *GSTP*1 polymorphisms to be significantly associated with gradual increase of severe malaria compared to mild malaria in children. Furthermore alpha^{+}- thalassemia polymorphisms have been found to confer protective effect against severe falciparum malaria. Lastly, this study concludes that, there is a slight inverse relationship between *GSTP*1 polymorphisms and alpha^{+}-thalassemia to children with severe malaria.

6.2 Recommendations

Further studies should be conducted in order to fully validate the relationship between GSTP1 polymorphisms and alpha+-thalassemia in children with severe malaria.

This also should include determination of prevalence in GST polymorphisms and alpha+-thalassemia in malaria endemic regions in the world.

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