THE UNIVERSITY OF ZAMBIA
SCHOOL OF MEDICINE

DEPARTMENT OF MICROBIOLOGY & PATHOLOGY

INFLUENCES OF HAEMOGLOBIN-AS GENOTYPE ON ASYMPTOMATIC PLASMODIUM INFECTIONS IN CHILDRED IN NCHELENGE DISTRICT, LUAPULA PROVINCE, ZAMBIA

A Dissertation submitted to the University of Zambia in Partial Fulfillment of the Requirement for the Degree of Master of Science in Pathology (Haematology)

By

CHIANZU GRAHAM PUMULO, BSc

STUDENT ID 512806943

Supervisor

Dr. Trevor Kaile

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DECLARATION

I, **Graham Pumulo Chianzu**, hereby declare that this dissertation represents my own work and that it has not been previously submitted for a degree, at this or any other University.

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Graham P. Chianzu

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Date

CERTIFICATE OF APPROVAL

This dissertation submitted by **Chianzu Graham Pumulo** has been approved as fulfilling part of the requirements for the award of the degree of MASTER OF SCIENCE IN PATHOLOGY (HAEMATOLOGY) at the University of Zambia.

Dr Trevor Kaile _____ _____

Supervisor Signature Date

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Examiner Signature Date

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ABSTRACT

Background: It is approximated that about 50% of malaria infections are asymptomatic in areas where malaria is endemic. In these areas, transmission is intense and consistent over time. As a result, most adults who live in these endemic areas possess partial immunity to malaria due to recurrent infections. Infants and children unfortunately, usually do not acquire this partial immunity early in life until they are exposed to malaria infection for a long time. These asymptomatic individuals continue transmitting the disease to others and provide a long-lasting reservoir for the malaria vector. It has been noted that there has been a higher prevalence of haemoglobin S in highly malaria endemic areas, especially in sub-Saharan Africa. And it has been reported that haemoglobin AS heterozygote [HbAS; sickle-cell trait] protects against severe disease & death due to *Plasmodium falciparum*. The aim of the study was to establish the effects of Sickle cell genotypes on asymptomatic malaria infection among children in Nchelenge district.

Method: Malaria parasites were counted per 200 white blood cells [WBCs] on Giemsa-stained thick blood films, in determining parasitaemia & parasite density we calculated assuming a mean WBC count of 8000/µL. Malaria was defined as any parasitaemia plus fever. Samples from all participants with RDT positive, and blood smear negative and all positive blood smears [for parasite identification] were subjected to PCR. DNA was extracted from dried blood spots by Chelex DNA extraction, and submicroscopic infections were ascertained by nested PCR assays including commercial negative and positive controls. We extracted DNA using a QIAGEN kit and haemoglobin was typed by polymerase chain reaction-restriction fragment length polymorphism.

Results: Microscopically visible parasitaemia was present in 35.9% (83) of the children, at overall geometric mean parasite density (4435.4/µL; 95%CI, 3292.5-5975.1/ µL). By PCR, *P. falciparum* occurred in 89% (104/116) while 11% (12/116) were other species of malaria. The HbAS trait was present in 24.4% (56/230) of the children, while 71.7% (165/230) had a normal haemoglobin genotype (HbAA). HbSS occurred in 3.9% (9/230) of the children. Children with HbAS had reduced parasite densities as compared to those with HbAA.

Conclusion: In conclusion, our data showed that sickle cell trait (HbAS) protects against high parasitaemia, parasite density [*P. falciparum*] and anaemia in children, through the enhancement of the acquired and innate immunity, which inhibits parasite proliferation.

DEDICATION

I dedicate this dissertation to my late Parents Phoebe Mwenyo & Teddy Chianzu, my beloved late sister Tessa Womba Chianzu and My wonderful wife Caroline and children Gray & Gwendolyn. Mom you were and will always be my driving force, Caroline (Sweetie), Gray and Gwendolyn you have sacrificed a lot for me to be where I am today may God bless you in all that you do.

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CONTENTS

LIST OF ABBREVIATIONS

%	: Percentage
Cl	: Confidence Interval
DBS	: Dry Blood Spots
DNA	: Deoxyribose nucleic Acid
G6PD	: Glucose 6 Phosphate Dehydrogenase deficiency
Glu	: Glutamic acid
Hb	: Haemoglobin
HbAA	: Normal haemoglobin genotype
HbAS	: Heterozygous for sickle cell disease (sickle cell trait)
HbSS	: Homozygous for sickle cell disease
ICEMR	: International Centers of Excellence for Malaria Research
NCBI	: National Centre for Biotechnology Information
NORMAP	: Northern Region Malaria Project
PCR	: Polymerase Chain Reaction

RFLP	: Restriction Fragment Length Polymorphism
RBC	: Red Blood Cell
RDT	: Rapid Diagnostic Test
TDRC	: Tropical Diseases Research Centre
UNZA	: University of Zambia
Val	: Valine
WHO	: World Health Organisation
WBC	: White Blood Cell

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CHAPTER ONE

1.1 BACKGROUND

1.1.1 Sickle Cell anaemia & malaria in sub-Saharan Africa

The greatest burden of sickle cell disease is in sub-Saharan Africa, where 75% of the 300,000 global births of affected children live (WHO 2006). Malaria remains a public health problem of overwhelming importance, with more than 300-500 million cases and one to two million deaths each year (Marsh, English et al. 1996; Hay, Smith et al. 2008; von Seidlein, Olaosebikan et al. 2012) in the same area. Malaria transmission is and still remains highest in Oceania and Sub-Saharan Africa (Singh, Kim Sung et al. 2004). It is approximated that about 50% of malaria infections are asymptomatic in areas where malaria is endemic (Nsobya, Parikh et al. 2004; Coura, Suarez-Mutis et al. 2006). In these areas, transmission is intense and consistent over time. As a result, most adults who live in these endemic areas possess partial immunity to malaria due to recurrent infections (Coura, Suarez-Mutis et al. 2006). Infants and children unfortunately, usually do not acquire this partial immunity early in life until they are exposed to malaria infection for a long time. Therefore, asymptomatic malarial infections are an important impediment to malaria control, because asymptomatic patients are not likely to seek treatment. These asymptomatic individuals continue transmitting the disease to others and provide a long-lasting reservoir for the malaria vector (Yeung, Pongtavornpinyo et al. 2004; Chiyaka, Garira et al. 2009). It has been known for some time now that some hereditary genetic disorders, such as sickle cell trait and G6PD also predispose people to experience asymptomatic malaria episodes (Vafa, Troye-Blomberg et al. 2008; Shim, Feng et al. 2012). Therefore, with the increased movement observed in human populations from rural areas to

bigger cities in sub-Saharan Africa, this high prevalence of asymptomatic infection increases the risk of malaria, particularly in malaria-free zones.

There has been a higher prevalence of haemoglobin S in highly malaria endemic areas, especially in sub-Saharan Africa. A number of investigators have reported that haemoglobin AS heterozygote [HbAS; sickle-cell trait] protects against severe disease & death due to *Plasmodium falciparum* (Williams, Mwangi et al. 2005; Billo, Johnson et al. 2012; Gong, Parikh et al. 2013). It is therefore obvious that a risk reduction at this level provides a survival advantage in a malarious environment. The high prevalence of the sickle haemoglobin gene [HbS], the result of a single point mutation [Glu→ Val] in the sixth codon of the globin chain in sub-Saharan Africa is generally thought to be because of the survival advantage conferred by its heterozygous form, known as sickle cell trait [HbAS]. Sickle-cell trait acts as a genetic modifier against malaria infection; this genetic modifier offered by HbAS genotype comes with a cost, as asymptomatic malaria is a major public health risk in general. A situation in which the majority of the population with asymptomatic malaria can then inadvertently act as a reservoir for transmission of malaria ends up increasing clinical malaria in the community and therefore defeats the fight against malaria, on which governments are expensing a lot of resources.

1.1.2 Malaria in Zambia and Nchelenge district

Malaria is endemic in Zambia and still remains a major public health problem and as a disease of poverty, and its transmission is stable (moderate to high) in most districts with a seasonal peak associated with rains from November to April. In areas of stable and relatively high transmission like Nchelenge, it has been reported that women and their newborn children are the ones that bear a high burden of malaria morbidity and mortality (Korenromp, Armstrong-Schellenberg et al. 2004; Nambozi, Malunga et al. 2014). There are epidemiological variations

in malaria prevalence countrywide with an increasing malaria transmission gradient from south-west to north-east regions of the country. The main parasite species is *Plasmodium falciparum* accounting for over 95% infections. *Anopheles gambiae* and *Anopheles funestis* are the main vectors.

1.2 STATEMENT OF THE PROBLEM

The reported higher prevalence of malaria in Nchelenge as compared to other parts of the country, particularly in children and women in the child bearing age group despite the interventions such as the Intermittent Preventive Treatment in pregnancy (IPTp), inspired major interest that we did this work.

1.3 JUSTIFICATION OF THE STUDY

The prevalence of malaria still remains high in some areas such as Luapula province of Zambia. Therefore we proposed to undertake a study to ascertain whether or not some Haemoglobinopathies such as sickle cell disease may be contributing to this high prevalence as noted in other endemic areas in West Africa.

As sickle cell trait is associated with lower malaria parasite densities and it is also a risk factor of asymptomatic malaria. HbAS and other related traits or genotypes have influences on the incidence of parasitaemia, uncomplicated malaria, anaemia, and possible effects on stunted growth, the marker for childhood development and a risk factor for childhood mortality during the first 5 years of life.

We set out to determine whether HbAS genotype is the risk factor for the effects of asymptomatic *Plasmodium falciparum* malaria among children in Nchelenge district.

1.4 RESEARCH QUESTION

Is HbAS and HbSS genotypes risk factor for asymptomatic *Plasmodium falciparum* malaria infection among children in Nchelenge district?

1.5 GENERAL OBJECTIVE

To establish the effects of Sickle cell genotypes on asymptomatic malaria infection among children in Nchelenge district.

1.6 SPECIFIC OBJECTIVE

1.6.1 To determine the prevalence of Haemoglobin AA, AS, SS in children in Nchelenge district.

1.6.2 To determine prevalence of asymptomatic malaria in children who are sickle cell gene carriers.

1.6.3 To establish a correlation between sickle cell genotype and asymptomatic malaria, anaemia & malaria parasite density in children.

1.6.4 To determine the prevalence of sub-microscopic infections and of *Plasmodium falciparum.*

CHAPTER TWO

2.1 LITERATURE REVIEW

2.1.1 Association of Sickle cell trait with anaemia

Sickle cell disease is a common hereditary haemoglobinopathy that occurs primarily in individuals of African descent (Robbins, Kumar et al. 2010).Haemoglobin is a tetrameric protein composed of two pairs of globin chains, each with its own Haeme group. Normal adult red cells contain mainly HbA ($\alpha_2\beta_2$), along with small amounts of HbA_2 ($\alpha_2\delta_2$) and foetal haemoglobin (HbF; $\alpha_2\gamma_2$). Sickle cell disease is caused by a point mutation in sixth codon of β-globin that leads to the replacement of a glutamate residue with a valine residue. The abnormal physiochemical properties of the resulting sickle haemoglobin (HbS) are responsible for the disease (Robbins, Kumar et al. 2010). HbS molecules undergo polymerisation when deoxygenated. Initially the red cell cytosol converts from a freely flowing liquid to a viscous gel as HbS aggregates form. With continued deoxygenation aggregated HbS molecules assemble into long needle-like fibers within red cells, producing a distorted sickle or holly-leaf shape. The presence of HbS underlies the major pathological manifestations such as chronic haemolysis, microvascular occlusions and tissue damage (Robbins, Kumar et al. 2010).

Even though HbAS is said to protect against severe disease and mortalities from falciparum malaria infection (Allison 1964; Aidoo, Terlouw et al. 2002), it is also associated with lower malaria parasite densities (Stirnadel, Stockle et al. 1999). In real sense this predisposes an individual to develop chronic anaemia, as parasites infect red blood cells chronically there will be an increased cellular disruption and haemoglobin digestion which leads to directly haemolysis of RBCs. Most parasitized cells have an increased osmotic fragility and lose deformability, they thereby become sequestered and destroyed within the spleen, at the same

time non-parasitized cells may then become sequestered within the spleen, causing a raised plasma volume which contributes to the development to anaemia. The chronically lower malaria parasite density, in most cases causes or may lead to elevation of malaria antigens which may attach to non-parasitized red cells. This will lead to haemolysis via a complement-mediated immune response; hence these children with lower malaria density will be anaemic in the long term; as anaemia is a frequent manifestation of malaria (Diallo, Doumbo et al. 2004).The pathogenesis of this type of anaemia is complex and is affected by both human and parasite determinants (Ekvall 2003). HbC is more susceptible to precipitation than HbA in erythrocytes (MacDonald and Charache 1982; Krause, Diakite et al. 2012), where the products of haemoglobin denaturation (hemichromes) that bind to band 3 are thought to contribute to the more frequent removal of erythrocytes from the bloodstream of AC individuals with malaria relative to AA individuals with malaria (Diallo, Doumbo et al. 2004). This might also be true for HbAS carriers.

2.1.2 Prevalence of Sickle Cell gene

The frequency of the S-gene is approximately 10% among populations in which malaria is endemic and exerts substantial selective pressure on the human genome due to its high mortality and morbidity rates (Kwiatkowski 2005). It has been suggested that, this selective pressure is believed to be responsible for the high prevalence of sickle cell disease in malaria-endemic regions, which is known as a balanced polymorphism. Approximately one third of all inhabitants of Sub-Saharan Africa carry the S-gene (Carter and Mendis 2002). As a result, about 200,000 infants are born with sickle cell disease in Africa annually, and in some areas of sub-Saharan Africa, up to 2% of all children are born with sickle cell disease (Aliyu, Kato et al. 2008).

2.1.3 Association of Sickle trait & Malaria

HbAS has been shown to offer 70%-90% protection against severe malaria and 50% protection against uncomplicated malaria compared with individuals not carrying the sickle haemoglobin gene .However, protective host traits may also influence the far more frequent asymptomatic infections and possibly with an overall larger effect (Gong, Maiteki-Sebuguzi et al. 2012). The unravelling potential effects of haemoglobinopathies at this level may contribute to a better understanding of malaria epidemiology, of the mechanism of protection, and the increasingly realized interaction of the Hb variants upon reappearance of parasitaemia or clinical malaria after antimalarial interventions (Danquah, Ziniel et al. 2010).

It has been documented that individuals who carry HbAS gene [sickle cell trait] have a reduced risk of suffering from symptomatic malaria infections, and that HbAS do not seem to affect the course of asymptomatic infections [HbAS does not protect against asymptomatic *Plasmodium falciparum* infection] (Marsh 1992; Stirnadel, Stockle et al. 1999; Williams 2006; Vafa, Troye-

Blomberg et al. 2008). However, there are some conflicting reports by a number of investigators who have reported that HbAS protects against severe disease and death due *Plasmodium falciparum* (Aidoo, Terlouw et al. 2002; Billo, Johnson et al. 2012)**.** Most studies that have reported that Haemoglobin S gene protects against malaria were done before Hb electrophoresis came in use [most of them relied on sodium metabisulfite, which does not tell anything about the genotype of the patient], but recent studies have been using Hb electrophoresis and molecular tools (Billo, Johnson et al. 2012), and there are more methodologies being used now to ascertain the exact genotype.

Some work done earlier which suggested that HbAS protected against *P. falciparum* infection were performed at hospitals and clinics and were therefore potentially confounded by the inclusion of symptomatic malaria cases (Raper 1955; Billo, Johnson et al. 2012). It has been said that the Hb S serves as the paradigm for balanced polymorphisms: whereas persons with HbSS have sickle cell disease and those with HbAS genotype are sickle cell gene carriers. It has been demonstrated that the HbAS gene has a reduced risk for uncomplicated malaria in children, because most of the HbAS carriers are highly associated with reduced parasite densities when compared with carriers of the β-globin wild-type [HbAA] (Stirnadel, Stockle et al. 1999). It has also been demonstrated that the formation of sickle shaped cells under low oxygen tension takes place more rapidly in malaria infected red blood cells compared to those red blood cells that are not infected (Luzzatto from H. Franklin Bunn 2013). There is enhanced Hb S polymerisation in AS RBCs due to the rapid oxygen consumption which accompanies metabolic activity of the intracellular parasite (Bunn 2013). These hypotheses are shown in figure 1.0, adapted from H. Franklin Bunn.

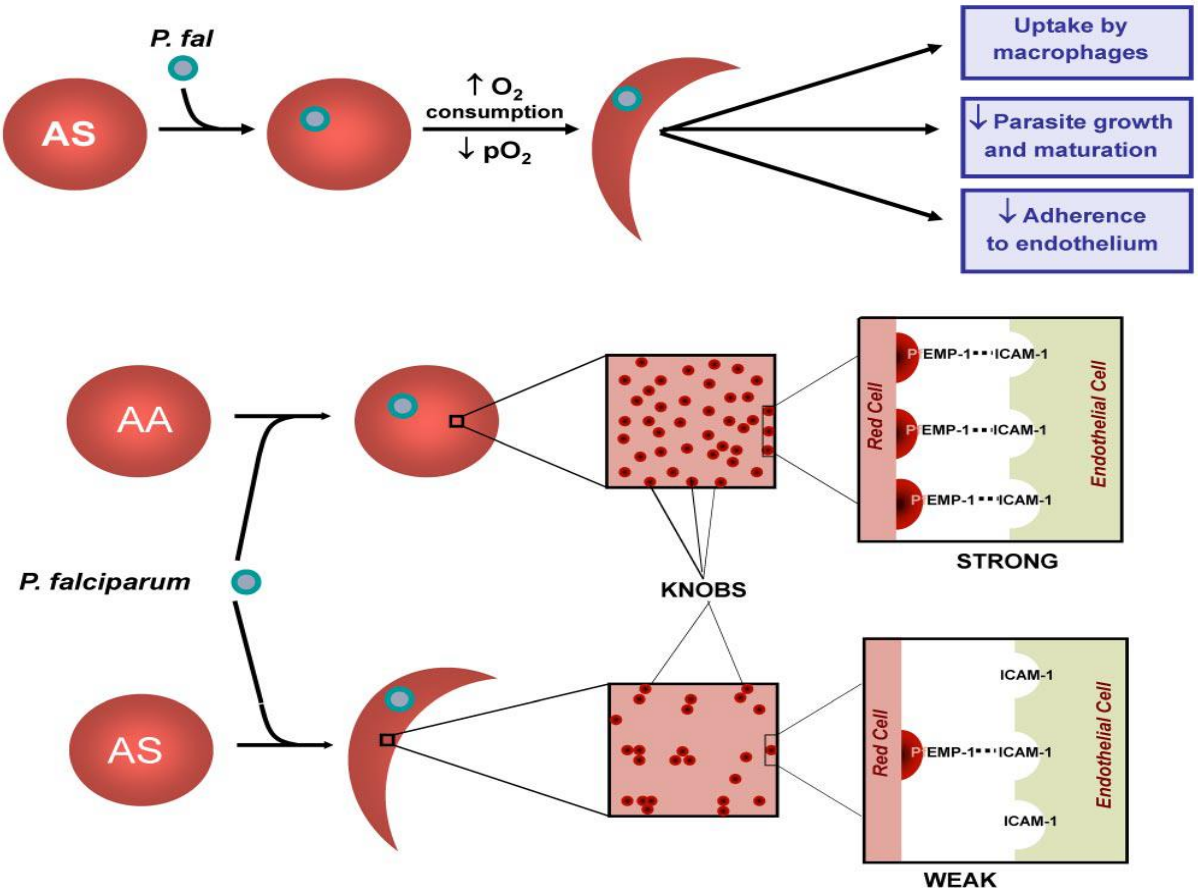


Figure 1.0 Mechanisms underlying protection by AS RBS against falciparum malaria. Parasitisation of AS RBCs causes increased oxygen consumption, a decrease in pO2, and sickle haemoglobin polymerization. The membranes of these cells are further modified by oxidant stress, resulting in uptake by macrophages, impaired parasite growth and development, and decreased adherence to endothelium. Parisitisation of AS RBCs leads to a decrease in the display of knobs of the cell surface along with uneven distribution. It is likely, though unproven, that hypoxia-induced sickling would aggravate this abnormal topology and further weaken interactions between the parasite protein PfEMP-1 and cognate receptors on endothelial cells, such as ICAM-1 in the brain.

2.1.4 Asymptomatic & Submicroscopic Malaria

Diagnosis of asymptomatic malaria is not straightforward due to the obvious lack of clinical manifestations and often undetectable by standard microscopy [subpatent parasitaemia] (Bottius, Guanzirolli et al. 1996; Omer, Khalil et al. 2011). Asymptomatic malaria in endemic regions has become a serious cause for concern as efforts are being stepped-up towards eliminating the parasite (Trape, Zoulani et al. 1987; Omer, Khalil et al. 2011; Nankabirwa, Brooker et al. 2014)). Particularly, sub-patent malaria (parasitaemia) is still transmissible and has complicated elimination of malaria in high transmission regions (Laishram, Sutton et al.

2012). It has been suggested that more than 90% of exposed individuals are likely to be infected with chronic asymptomatic malaria (Trape, Zoulani et al. 1987; Nankabirwa, Brooker et al. 2014).

In a study done in Ghana, it was found that HbAS did not affect the risk of *P. falciparum* infection per se but was rather associated with a reduced proportion of microscopically visible parasitaemia and an increased one of submicroscopic infections (Danquah, Ziniel et al. 2010). Figure 1.1 shows the proportion of asymptomatic malaria infection and submicroscopic malaria infection in both high and low transmission settings. This is consistent with a suppression of parasite density to levels less than the microscopy threshold and reduced parasite densities were observed in asymptomatic children with the sickle cell trait in the NORMAP study (Danquah, Ziniel et al. 2010).Haemoglobinopathies such as sickle cell diseases are well known to protect from the most dramatic and fatal manifestation of Plasmodium infection, such as severe malaria (Aidoo, Terlouw et al. 2002; Williams, Mwangi et al. 2005; May, Evans et al. 2007). It is obvious that a risk reduction at this level provides a survival advantage in malaria endemic environment and this may also influence the far more predominantly asymptomatic malaria infections with an overall larger effect to the transmission of the parasites in the household and community at large. It thus seems conceivable that a reduced parasite load in asymptomatic infections may contribute to the observed lower incidence of malaria in individuals with HbAS (Williams, Mwangi et al. 2005).

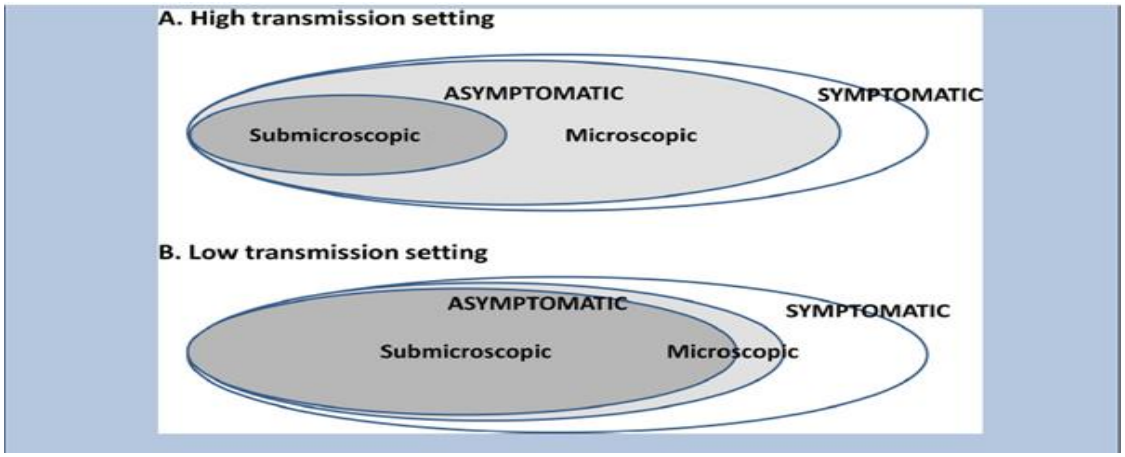


Figure 1.1. Showing proportions of asymptomatic malaria infection and Submicroscopic malaria infection in both low and high transmission settings

CHAPTER THREE

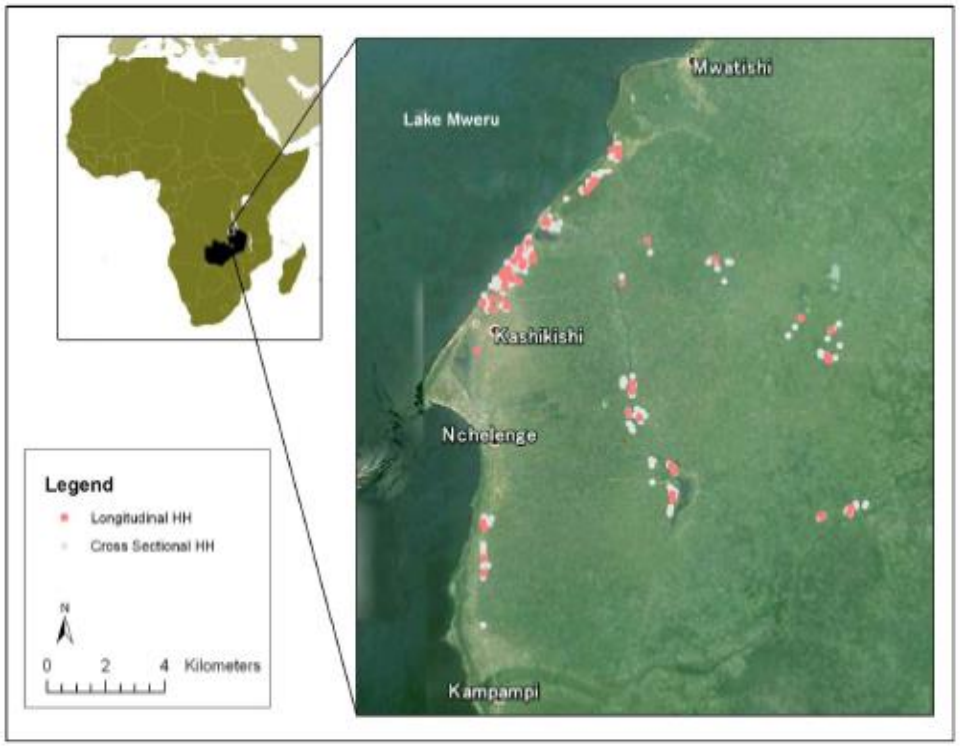
3.1 METHODOLOGY

3.1.1 STUDY DESIGN

This was a retrospective study which involved analysis of some data and samples from the Malaria Transmission and the Impact of Control Efforts in Southern Africa (ICEMR) Study, a household based study conducted from November, 2013 to June 2014.

3.1.2 STUDY SITE

The study was conducted in communities of Nchelenge district, Luapula province and the samples were analysed at TDRC, Ndola, Zambia, in the Haematology and Molecular Biology Units, Department of Biomedical Sciences.



Source: "Nchelenge" -9.343724° 28.787800° **Google Earth.** August 4, 2013. March 9, 2014
CIA The World fact Book: Zambia https://www.cia.gov/library/publications/the-world-factbook/geos/za.html

Figure.3.0, Map showing the location of Nchelenge in Zambia, with the dots showing the households where samples were collected

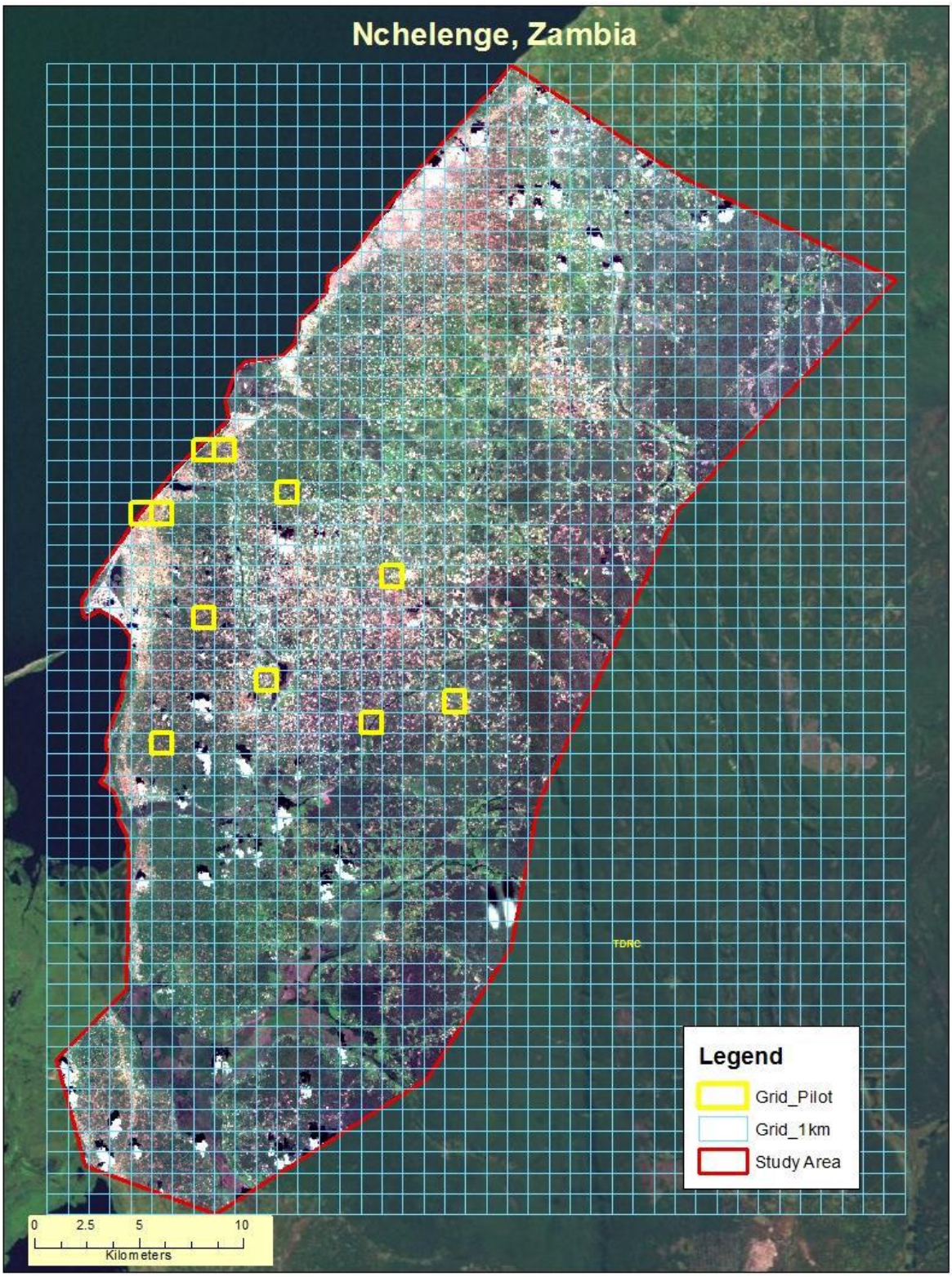


Figure.3.1, Nchelenge imagery showing grid plots in yellow samples were selected. Image provided by Dr. Tamaki Kobayashi

Nchelenge district

Nchelenge is one of the seven districts in Luapula province, north of Zambia. It lies between -9° (latitude) and 28° (longitude) with an elevation of 919m above sea level. According to the 2010 Zambian census report the population of Nchelenge district stands at 174, 000. The district has a total surface area of 4,793 square kilometers of which 60% is island, 10 % swamps and 30% is water. The district shares borders with Chienge district in the north, Kaputa in the north-east, Kawambwa district in the South east and Congo DR in the west. The Lake Mweru marks the boundary between Nchelenge and Congo DR. The inhabitants of Nchelenge are mostly fishermen and peasant farmers. The district has one first level referral hospital [St Pauls Mission hospital] with ten rural health centres and three health posts. Malaria prevalence in Nchelenge stands at 44.7% of all Out Patients Department cases in the RHCs and 80% of deaths recorded due to malaria with the most affected being children under 5 and pregnant women.

3.1.3 STUDY METHODS

Random sampling of households: High resolution satellite images of the study area were used to establish the sampling frame and randomly selected the study households. The images were of Natural Color, Ortho Ready Standard set to the appropriate UTM WGS84 projection with a ground sample distance of 0.64 m or better. It was ensured that optimal images were obtained when cloud cover was minimal. The image for Nchelenge District was obtained in 2010. Using ArcGIS software from ESRI™ (Redlands, CA), locations of all households within the study area was identified from the satellite image. Households were enumerated manually by placing a marker on the centroid of each potential residence, creating an attribute table containing unique identifiers and geographic coordinates for each household. A list of randomly-selected households were merged into data tables in ArcGIS, allowing visualization

of sampled and non-sampled households. When areas at high risk for malaria were identified, they were added to cluster sampling in the high risk areas to include more households where malaria transmission is highest. Households within the high risk area were randomly selected and neighboring households were also eligible to participate. Households that refused to participate were replaced (Agre 2010). The study included children aged six months to nine years, and was a minimal risk study.

3.1.3.1 SAMPLING PROCEDURE

The samples were retrieved from a previous study repository for children aged 6 months to 9 years; participants who met the inclusion criteria were recruited into the study. The samples were selected from the malaria transmission and impact of control efforts in Southern Africa study by an simple random sampling using Microsoft excel.

In Zambia, malaria transmission occurs throughout the year, with a peak during the rainy season between November and April, and it peaks in April-May and falls off in June-July when the rains stop. In the study Nchelenge district transmission period is 7 months as shown in figure 3.2 on the next page.

Sample selection was done from those samples that were collected between the months of November and May of each respective year of the study. For each participant selected some demographic data was collected, a blood slide, dried blood spot, and these were the samples we analysed. Microscopy was used to examine these slides and PCR for Hb genotyping and submicroscopic detecting. The results were matched for age and sex. On the next page is the map of Zambia showing the transmission of malaria for different regions.

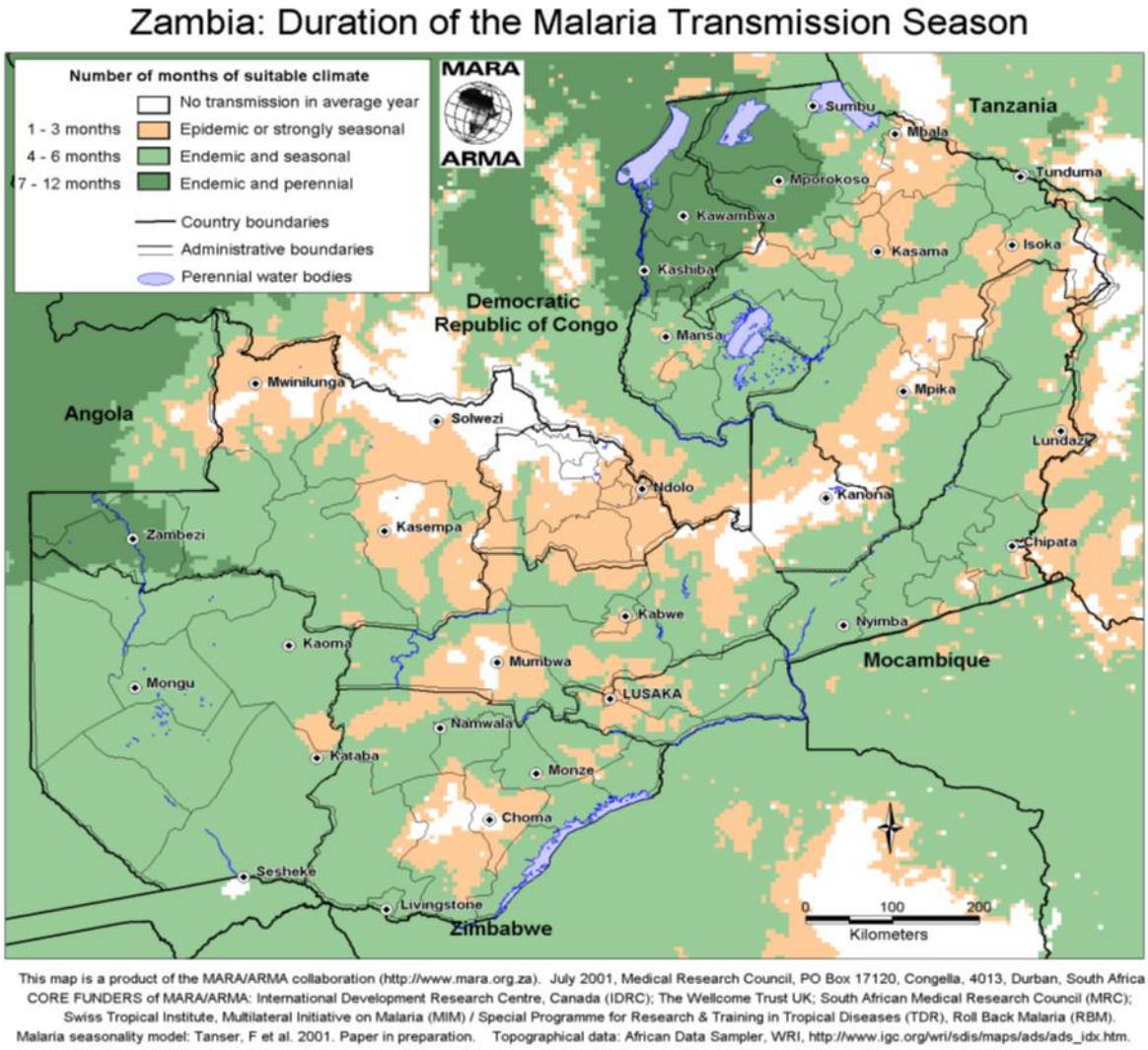


Figure. 3.2, Map of Zambia showing the Malaria seasonality model for different regions (Product of the MARA/ARMA collaboration).

3.1.4 SAMPLE SIZE

Based on an expected Sickle cell trait prevalence of 20% which is the observed prevalence among the population in Sub-Saharan Africa (Grosse, Odame et al. 2011), we needed to enrol 246 participants in order to identify the true prevalence with precision of +/-5% and 95% confidence interval.

$$N = \frac{Z^2 \times P(1-P)}{d^2} = \frac{1.96^2 \times 0.2(1-0.2)}{(0.05)^2} = 246$$

N=sample size z=statistic for 95% 1.96 p=expected prevalence 20% d=0.05

3.1. 5 TESTING PROCEDURE

3.1.5.1 HB LEVEL DETERMINATION

Hb concentrations of each participant was measured by a HemoCue photometer (HemoCue AB, Ångelholm, Sweden), and anaemia was defined according to age as shown in table 3.1. Hb concentration was already determined for all the participants.

Table 3.1. Haemoglobin levels to diagnose anaemia by age

		Anaemia*		
Population	Non-Anaemia*	Mild^	Moderate	Severe
Children 6 - 59 months	10.0 or higher	10.0-10.9	7.0-9.9	lower than 7.0
Children 11 - 10 years of age	11.5 or higher	11.0-11.4	8.0-10.9	lower than 8.0

* Haemoglobin in grams per declitres

^ "Mild" is a misnomer: iron deficiency is already advanced by the time anaemia is detected. The deficiency has consequences even when no anaemia is clinically apparent.

3.1.5.2 MALARIOMETRIC INDICES

Malaria parasites were counted per 200 white blood cells [WBCs] on Giemsa-stained thick blood films, in determining parasitaemia & parasite density we calculated assuming a mean WBC count of 8000/µL. Herein, 'parasitaemia' refers to a positive result on expert microscopy. Malaria was defined as any parasitaemia plus fever.

3.1.5.3 SUBMICROSCOPIC DETECTING & PLASMODIUM SPECIES

All samples from participants with RDT positive, and blood smear negative and all positive blood smears [for parasite identification] were subjected to PCR. DNA was extracted from segments of bloodspots on filter paper by Chelex DNA extraction, and submicroscopic infections were ascertained by nested PCR assays including negative and positive controls.

3.1.5.4 HAEMOGLOBIN GENOTYPING

We extracted DNA using a QIAGEN kit [QIAamp DNA blood mini kit] and the haemoglobin was typed by polymerase chain reaction-restriction fragment length polymorphism [PCR-RFLP]. And briefly, DNA samples were amplified by using a 5'-AGG AGC AGG GAG GGC AGGA-3' forward primer and a 5'-TCC AAG GGT AGA CCA CCA GC-3' reverse primer. The 358 base pair bp fragment was obtained by digestion with MnlI restriction endonucleases so that we could discriminate between HbAA [173 bp, 109 bp, and 60bp], HbSS [173 bp, 109 bp, and 76 bp] and HbAS [173 bp, 109 bp, 76 bp and 60bp]. A second digestion was done with DdeI restriction endonucleases which allowed for further discrimination for ambiguous results between HbSS [331 bp] and HbAS [130 bp, 201 bp and 331 bp]. All digestion were carried out for three hours [or more than 3hrs but not more than 16hrs] at 37°C and the PCR products were run on 3% agarose gel (Modiano, Luoni et al. 2001; Bougouma, Tiono et al. 2012).

3.2 ETHICS STATEMENT

3.2.1 ETHICAL CONSIDERATION

Ethical approval was sought from the UNZA Biomedical Research Ethics Committee [UNZABREC] prior to the initiation of the study. Permission to conduct the study in the department of Biomedical **S**ciences and to use the ICEMR samples (an on-going malaria epidemiological study), was sought from the Director of TDRC. Samples were collected with the explicit consent of the 'participants' parents/caregiver for analysis & storage.

3.2.2 SUBJECT CONFIDENTIALITY

Participants' confidentiality was strictly kept by the investigator, the staff involved, and the sponsor and their agents. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participates. The study protocol, documentation, data and all other information will be held in strict confidence. No

information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor and the UNZABREC.

3.2.3 INFORMED CONSENT PROCESS

This study did not seek consent from study participants because the 'participants' parents/caregiver had given their explicit consent for samples to be stored and used for further analysis. Some of the clinical data was extracted from the files.

There is no benefit or risk to participants. The benefits are to the community.

3.3 DATA ANALYSIS

Data entry and cleaning of both the socio-demographic data and laboratory results was done using Epi-Info Version 7.0; in a double-entry process incorporating range and consistence checks. The primary statistical package to be used for analysis is STATA Version 11.0/ SE. However, where necessary the data was subjected to conversion to other statistical software for some specific analyses.

Descriptive analyses; means and standard deviations for continuous variables and frequency distributions for categorical variables was determined for both the participants' demographic data and laboratory results.

In the analysis of the epidemiological data, malariological and haematological parameters were compared between groups defined by their haemoglobin genotype. Malaria Parasite densities were normalised by the $\log_{10}10$ transformation, and geometric mean parasite densities [GMPDs] along with their 95% confidence intervals [CIs] were calculated. All continuous variables were compared between groups by the Student's t test, analysis of variance [ANOVA], or by Mann-Whitney U test and proportions were done by Chi-square test or

Fisher's exact test as applicable. Odds ratios [ORs] and 95% CIs were computed. Bivariate and multivariate analysis were performed by logistic regression models to examine the relationship between *P. falciparum* infection and potential risk factors.

The validity, reliability and efficiency were evaluated comparing different diagnostic technics for malaria such as microscopy, RDT and PCR.

CHAPTER FOUR

4.15 Results

The mean age of the 231 children [114 girls, 117 boys] was 53 months [range, 7 months to 9 years] 66.2% [153] were under the age of five years. Overall, all the children lived in villages surrounding Kashikishi in Nchelenge.

Table 4.1. Baseline characteristics of the 230* children from Nchelenge in Luapula Province

	Haemoglobin genotype		
	AA	AS	SS
No (%)	165 (71.7)	56 (24.4)	9 (3.9)
Proportion of girls (%)	52.1	42.9	33.3
Age (months; mean, range)	49.7 (7-108)	61.6 (8-108)	58.6 (11-108)
Axillary temperature(˚C; Mean±SD)	36.9 ± 0.5	37.0 ± 0.5	36.9 ± 0.5
Proportion febrile (%)	1.2	5.4	0
Hb (g/dL; mean,range)	10.5 (4.5-14.4)	10.4 (4.3-15.1)	10.8 (8.4-13.6)
Hb>11g/dL-No. (%)	67 (40.6)	22 (39.3)	4 (44.4)
Hb<11g/dL-No. (%)	65 (39.4)	25 (44.6)	4 (44.4)
Hb< 9g/dL-No. (%)	27 (16.4)	6 (10.7)	1 (11.1)
Hb< 7g/dL-No. (%)	6 (3.6)	3 (5.4)	0 (0)

* One Child had information on sex missing, so when we stratified for sex. The child was dropped from the analysis.

4.1.1. Malariometric parameters

Overall the number of children who were positive by the rapid diagnostic test was 60.8% and by microscopy the prevalence of malaria was 35.9% with PCR giving 39.2%. Results for RDT were twice higher as that of microscopy giving about 24.9% false positives. The difference between PCR and microscopy was not significant. It was noted that Plasmodium infections increases with/by age [older children were mostly infected than the younger ones] as shown in figure 4.0 regardless of the diagnostic method/technical.

The geometric mean parasite densities were lower for children with HbAS genotype [2548.7 parasites/µL] than that of the children with Hb wild type with 6041.2 parasites/µL and it was

much lower in children who were less than 2 years old as it is shown in figure 4.1. Which is also supported in figure 4.5, which showed that the proportion of children with HbAA genotype had a higher parasitaemia as compared to HbAS genotype.

Overall, anaemic children had a similar prevalence of microscopically visible parasitaemia 66.2% [55/83] or of PCR-detected P. falciparum infection 66.7% (60/90), as compared to the non-anaemic children [33.7%, 28/83; and 33.3%, 30/90 respectively for malaria negative and positive].

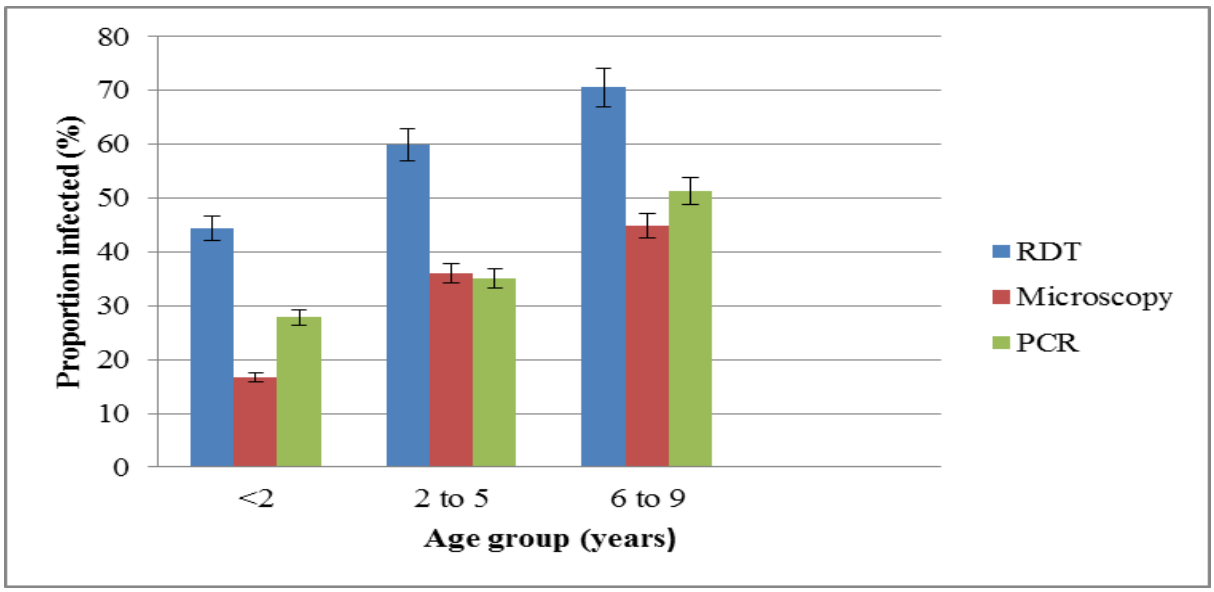


Figure 4.0. Percentage of Plasmodium infection according to age group and diagnostic method <2 years, 32; 2 to 5 years, 153; 6 to 9 years, 130.

The proportions of infected children by age group and diagnostic methods, revealed that RDTs gives a lot of positive malaria results than PCR and microscopy. It can also be seen that the proportion of malaria infections increases with age.

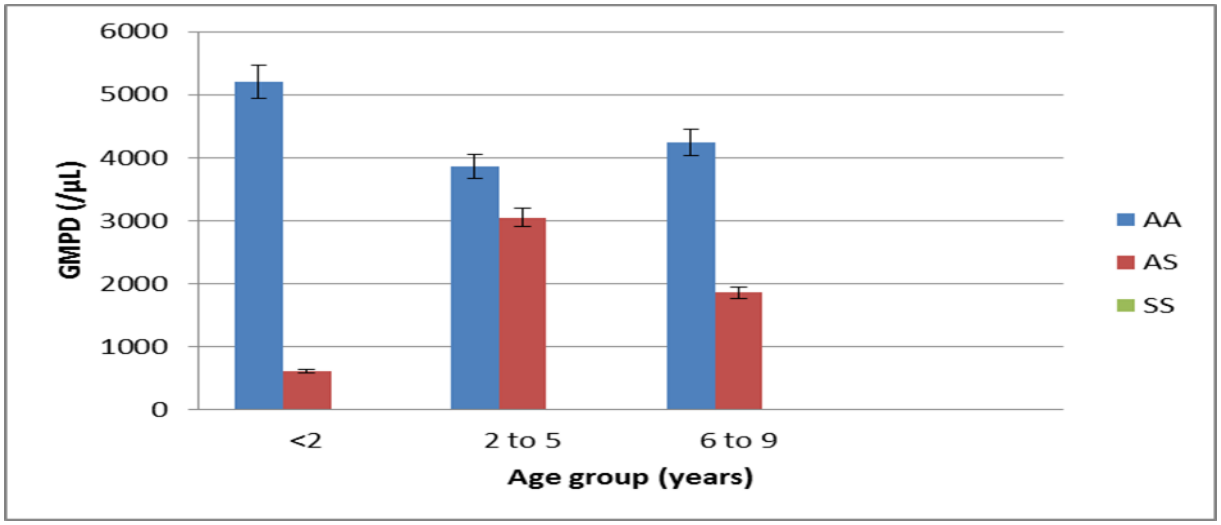


Figure 4.1, Geometric mean parasite density [GMPD] according to age and haemoglobin genotype

Group sizes: 0<2 years, 36; 2 to 5 years, 117; ≥6 years, 78
Error bars represent 95% Cls for GMPD

It was noted that Hb genotype influenced the geometric mean parasite densities of Plasmodium parasites, as it can be seen that children with HbAS has a decreased GMPD than those with HbAA in all the age groups.

4.1.2. Anaemia

It was noted that Hb level improves with age as is shown in figure 4.2, and it was not affected by Hb genotype as shown in figure 4.4. The average haemoglobin concentration was 10.5 g/dL. More than 50% of the children had an Hb level of < 11 g/dL overall in the study [table 4.1]. The Hb means were as follows 10.5g/dL [95% CI 10.2-10.7] for HbAA; 10.4 g/dL [95% CI 9.9-10.9] for HbAS; and 10.8g/dL [95% CI 9.4-12.2] for HbSS. While, the overall mean Haemoglobin concentration was 10.5 g/dl [range, 4.3-15.1 g/dl] and increased with age P< 0.0001. All the children with severe anaemia [ranging from 4.3-6.9g/dL] were under the age 5 years. The influence of parasitaemia on Hb levels was clearly seen [*F*=1.83]. The overall rate of anaemia was 59.4%, with severe anaemia [<5 g/dl] affected two children only [0.01%].

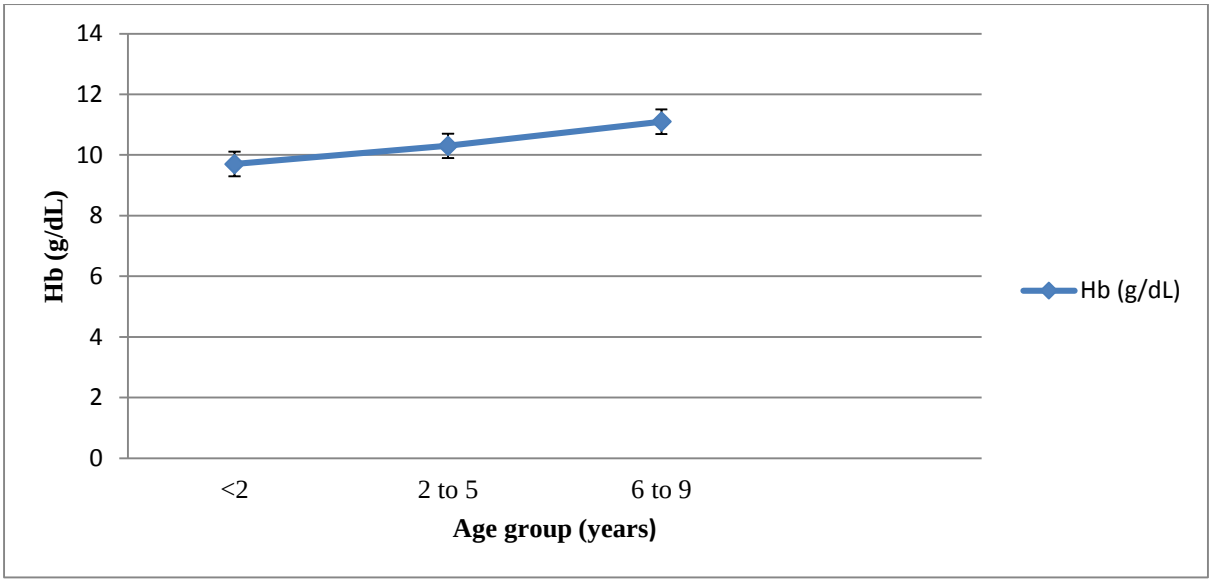


Figure 4.2. Mean haemoglobin [Hb] concentrations according to age Group sizes: <2 years, 35; 2 to 5 years, 116; 6 to 9 years, 78
Error bars represent standard errors for mean Hb.

It was seen that the concentration [level] of Hb increases as the children grows older [increases with age].

4.1.3 Haemoglobin variants

Hb genotype was not significantly different with age or sex. The prevalence of the HbAS genotype was 24.4% and sickle cell disease [HbSS] occurred in 3.9% of the children as summarised in Table 4.1. Most of the children across all age groups had the wild type [HbAA] as compared to HbAS/SS. Anaemia was more frequent in children with HbAS than those with HbAA/SS, but the Hb concentration were generally lower for most of the children regardless of the Hb genotype. Children with HbAS had a lower prevalence' of parasitaemia [P<0.001], and as well as a higher proportion of submicroscopic *P. falciparum* [P=0.272]. For HbSS, there was no statistically significant finding as shown in Table 4.2 and 4.3.

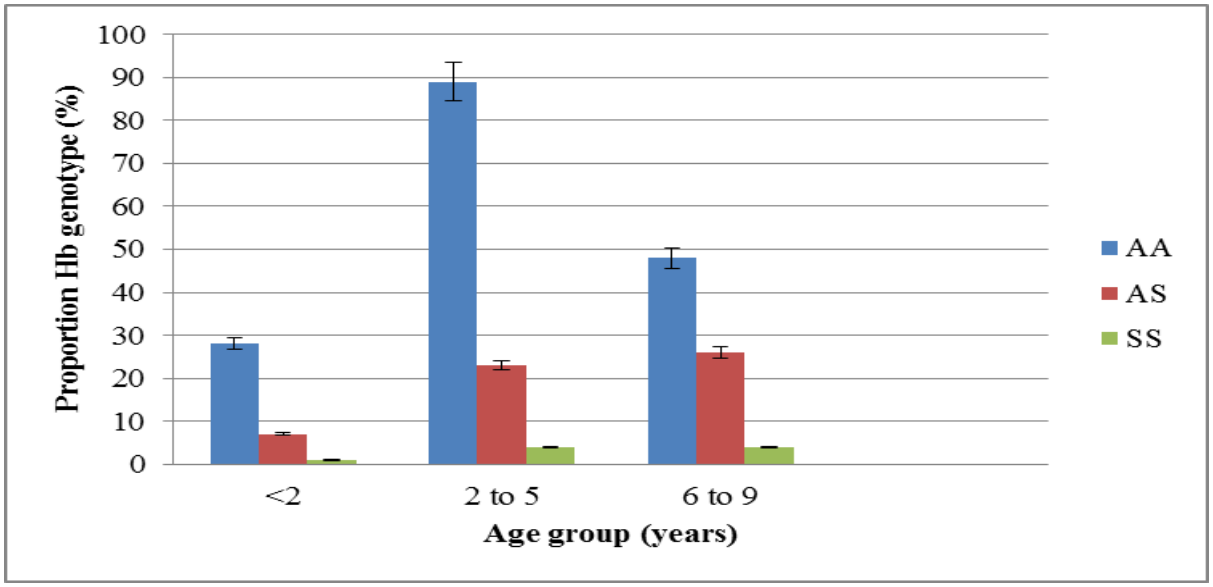


Figure 4.3, Proportion of Hb genotype according to age Group sizes: <2 years, 36; 2 to 5 years, 117; 6 to 9 years, 78
Error bars represent 95% Cls for proportion of Hb genotype.

The most common Hb genotype is the wild type [HbAA] in the various age groups. Followed by AS and SS is only found in low percentage. Generally, the levels of Hb across the three genotypes [AA, AS & SS] was not different as shown in figure 4.4, the mean was similar with the P<0.0001.

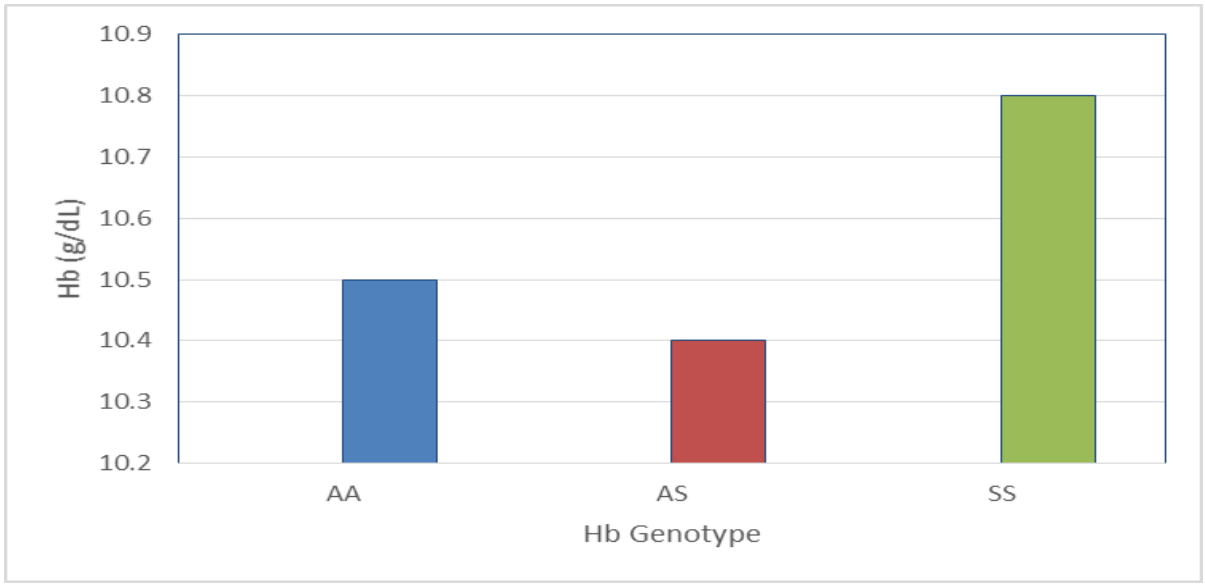


Figure 4.4. Hb concentration according to haemoglobin genotype: Size AA, 165; AS, 56 and SS, 9.

It was seen that children with HbAA had a higher Hb mean than those with HbAS. Children with HbSS gave a higher Hb mean [10.8 g/dL] than the other genotypes but this can't be taken

to be true or compared to others because of the fewer number of children with the HbSS genotype [only 9 children].

The results revealed that Hb genotype influenced parasitaemia of Plasmodium parasites, as it was observed that children with HbAS had a lower parasitaemia than those with HbAA in all the age groups.

Table 4.2. Parasitological indices according to Hb genotype

	Haemoglobin genotype		
	AA	AS	SS
No (%)	165 (71.7)	56 (24.4)	9 (3.9)
Positive by RDT (%)	58.8	78.6	0
Parasitaemia, microscopy (%)	63.9[a]	36.2	0
Parasite density >1000/µL (%)	21.3[a]	9.6	0
GMPD (95%CI)	6041.2 (4161.2-8770.4)[a]	2548.7(1617.8-4015.2)	0
Proportion submicroscopic	12.0[a]	39.2	0
P. falciparum infection, PCR (%)	27.3	80.4	11.1
All	31.0	88.2	12.5
Febrile parasitaemia (%)	1.5	1.7	0

[a]across all groups, $P<0.005$ by x^2 test

Table 4.2 Shows that children with HbAS genotype had a higher proportion of Submicroscopic malaria infection at 39.2% than those with the wild type which was 12.0%. It summarises all malaria diagnostic parameters, it also reveals that all malaria indices for children with HbAS genotype had a decreased values for parasitaemia, parasite density and geometric mean parasite density.

Severe anaemia was only seen among the under 5 years children of the Hb AA and AS with the percentage not significantly different as shown in table 4.3. Children with HbAS genotype had a higher proportion of severe anaemia at 6.9% compared to HbAA genotype at 4.3%.

Table 4.3. Frequency and severity of anaemia among [Children] cases stratified by age group and Hb genotype

	Under 5 years			5 to 9 years		
	AA	AS	SS	AA	AS	SS
No (%)						
Mild anaemia (Hb9 -11g/dL)	116 (42.2)	29 (51.7)	5 (60.0)	48 (35.4)	26 (50.0)	4 (25.0)
Moderate anaemia (Hb<9g/dL)	116 (19.0)	29 (20.7)	5 (20.0)	48 (10.4)	26 (0)	4 (0)
Severe anaemia (Hb<7g/dL)	116 (4.3)	29 (6.9)	5 (0)	48 (0)	26 (0)	4 (0)

Table 4.3 reveals that when stratified by age Hb genotype influences Hb concentration, i.e. HbAS protects against moderate and severe anaemia in children between 5 to 9 years than those with under 5 years.

CHAPTER FIVE

5.1 Discussion

The overall prevalence of malaria in the children was found to be 35.9%, which was similar to previously reported estimates of prevalence in a study done in the same area, which was 30.2% (Nambozi, Malunga et al. 2014) but three times higher than what was reported, in 2008 where the national average was 10.3% (MOH 2008) which classifies Nchelenge as an area of intense malaria transmission. The national survey conducted in 2010 reported a higher resurgence of malaria in Luapula province than other provinces (MOH 2010). This could be attributed to human genetics or environmental factors. We reported a higher geometric mean parasite density [GMPD] of 4435.4 parasite/µL [95%CI, 3292.5-5975.1/ µL], than in a previous study done by Nambozi in the same area. This high GMPD was in children, where most of them were asymptomatic and was relatively higher in 2 to 4 years age group. By PCR, *P. falciparum* occurred in 89% [104/116] while 11% [12/116] were other species of malaria. With, overall submicoscopic malaria infections at 14.5% which was classified only as positive by PCR, which were cases missed by routine malaria diagnostic tool [microscopy]. Fever was present in 1.72% [4/231] and febrile parasitaemia in only 1.3% [3/231] of the children. With age, parasitaemia and PCR-detected infections *Plasmodium* species increased in prevalence [each, $P< 0.002$], whereas a trend only was seen for mean parasite density by microscopy. The age of a child was found to be an important factor with increased odds of parasitaemia and parasite density [OR, 1.014; 95% CI, 1.005-1.023 and OR, 1.013; 95% CI, 1.004-1.023] respectively. Younger children usually have lower parasitaemia, because of the protection they get from maternal antibodies.

The prevalence of HbAS in the study population was 24.4% (56/230) which is similar to the observed prevalence among the population in Sub-Saharan Africa (Grosse, Odame et al. 2011).

12.9% of the HbAS carriers were positive for malaria microscopy and 39.2% Submicroscopic, which is consistent with the hypothesis that HbAS lowers the parasite densities below the threshold for microscopy. The distribution of the Hb genotype did not deviate from the Hardy-Weinberg equilibrium, and no significant differences with respect to age or sex were observed. HbSS occurred in 4% of the children. Parasitological parameters for children with HbAS showed reduced parasite densities as compared to the ones with HbAA. Proportion of febrile parasitaemia did not differ with Hb genotypes although malaria was not observed in the group of children with HbSS. When children with febrile parasitaemia were excluded from the above calculations it did not basically change the results. Children with the wild-type Hb, with the parasite density of >1000 parasites/μL were as twice higher than the HbAS, due to the fact that plasmodium parasites are subjective to poor growth under low-oxygen tension; and accelerated acquisition of antibodies specific for *P.falciparum* in red blood cells with HbAS. Febrile parasitaemia was similar in both groups.

We found a very low prevalence of gametocytes in this study, this could be attributed to the method we used which was microscopy. Examining thick blood smears for gametocytes, especially when they are present at low densities, it has been reported to be a challenge even for experienced microscopists (Karl, Davis et al. 2009). Therefore, microscopy can miss most of the malaria sexual stage which has also been reported by others (Warhurst and Williams 1996; Bejon, Andrews et al. 2006; Babiker, Schneider et al. 2008). This was one of the limitation of our study in which we used thick smears to identify gametocytes; if we had used molecular tools to identify gametocytes the prevalence would have possibly been higher.

Haemoglobin disorders (Haemoglobinopathies) such as HbS have be well known for over 50 years to protect people affected from the most severe malaria infections (Danquah, Ziniel et al. 2010). The risk reduction offered at this level gives a survival advantage in malaria endemic areas. But the protective host traits may also influence the more frequent asymptomatic malaria

infections. In this study, children between 6 months to 9 years of age living in a high transmission region of Zambia [Luapula province, Nchelenge district], HbAS genotype was found to be associated with lower risk of clinical malaria relative to HbAA genotype among children aged 0.5 to less than 2 years, the lower risk which we defined, has protection against symptomatic infections in malaria endemic areas, provided by HbAS has also been reported by other studies ((Fleming, Storey et al. 1979; Marsh 1992; Le Hesran, Personne et al. 1999; Stirnadel, Stockle et al. 1999; Aidoo, Terlouw et al. 2002; Bougouma, Tiono et al. 2012), starting with hall mark works by Beet, Allison and the team more than five decades ago (Beet 1946; Beet 1947; Allison 1954; Billo, Johnson et al. 2012). These results suggest that HbAS genotype should be considered as a potentially important factor, when evaluating febrile malaria risk in endemic areas among infants. The mechanisms by which HbS gene offers protection against malaria has been the matter of speculation for more than five decades. Mechanisms such as the decreased red cell invasion by the Plasmodium parasite or poor growth under low-oxygen tension; and accelerated acquisition of antibodies specific for *P.falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1) and other variant surface antigens (Bougouma, Tiono et al. 2012).

Our results showed that infants with HbAS genotype were protected against uncomplicated malaria and had a lower geometric mean parasite density of 2548.7 parasites/µL [95% CI 1617.8-4015.2], as compared to the wild-type with 6041.2 parasites/µL [95% CI 4161.2-8770.4].

Most probably as a result of the protection against periods of high parasitaemia, indirectly HbAS carriers should have less hospital admissions due to malaria. Because they rarely have episodes of fevers and they do not have malaria symptoms and do not go to hospital to seek treatment for malaria.

The observed lower parasite density and higher asymptomatic malaria prevalence in the children with the AS trait is similar to findings in some previous studies (Allen, Bennett et al. 1992; Lell, May et al. 1999; Stirnadel, Stockle et al. 1999; Aidoo, Terlouw et al. 2002). The decreased parasitaemia indicates that the HbS gene should directly or indirectly inhibit the invasion, presence, or proliferation of malaria parasite in the red blood cells (Kreuels, Kreuzberg et al. 2010). In the laboratory, it has been demonstrated that invasion of erythrocytes with HbAS by the Plasmodium parasite is reduced (Pasvol, Weatherall et al. 1978; Taylor and Fairhurst 2014) [which is mainly conferred by the physical properties of HbS red blood cells] an inhibition of parasite growth (Friedman 1978; Shear, Roth et al. 1993; Gupta and Gupta 2014) and some studies done on this matter has suggested that HbAS may also enhance the acquisition of the naturally acquired immunity (Cornille-Brogger, Fleming et al. 1979; Williams, Mwangi et al. 2005; Verra, Simpore et al. 2007). With the innate retention of the young and ring infected RBCs in the spleen which has been known to lower parasites densities (Safeukui, Correas et al. 2008). This role may be greater in HbAS traits possibly leading to lower parasite densities. The lower GMPD associated with HbAS which we reported is similar to findings in previous studies (Willcox, Bjorkman et al. 1983; Stirnadel, Stockle et al. 1999; Modiano, Bancone et al. 2008) as protection against symptomatic infection offers an innate control which is consistent with several findings by previous studies.

Although anaemia aetiologies are multi-factorial, several previous studies have been confined to anaemia associated with malaria and other individual factors, but in this study we wanted to investigate the role which Hb genotype play in children. Our results support, the hypothesis that HbS protects from anaemia, which has also being reported by some previous studies (Kreuels, Kreuzberg et al. 2010). Therefore, because the major cause of anaemia in children in rural areas in Zambia [sub-Saharan Africa] is chronic malaria and recurrent infections. Chronic anaemia is one of the major cause of morbidity in young children in sub-Saharan Africa

(Ehrhardt, Burchard et al. 2006; Kreuels, Kreuzberg et al. 2010). We found that children with malaria had lower Hb concentration {9.7g/dL [95% CI 9.3-10.3]} than those without malaria {10.7g/dL [95% CI 10.5-11.0]}. The rate of anaemia between children with malaria and without malaria was not all that significant. This means that the protection against anaemia by HbS may give an indirect survival advantage. Therefore, we can say that major causes of anaemia in children such as malnutrition (de Silva 2003) may have played a big role in what we saw and malaria was just the other cause. And we also say that other parasites such as intestinal parasites [worms] may have been a confounder in the anaemia we saw, which induce an inadequate intake of macro and micronutrients, or intestinal malabsorption, which leads to iron and folate deficiency, a well- documented role in chronic anaemia pathophysiology (Khieu, Odermatt et al. 2006).

The results of this study suggest that the prevalence of HbAS in a population may not only reduce the incidence of severe malaria, which has been suggested before by earlier studies (Pasvol, Weatherall et al. 1978; Le Hesran, Personne et al. 1999; Kwiatkowski 2000), but it may also increase or maintain a steady rate of transmission due to the chronic parasitaemia seen in the asymptomatic human population in these highly malaria endemic areas.

CHAPTER SIX

6.1 CONCLUSION, RECOMMENDATIONS AND STUDY LIMITATION

6.1.1 CONCLUSION

In conclusion, our data showed that the most common Hb genotype is the wild type (HbAA) standing at 71.7%, followed by HbAS at 24.4% and 3.9% of the children had HbSS. The prevalence of malaria by the type of diagnostic methods was different with RDT, PCR and microscopy at 60.8, 39.2 and 35.9% respectively. With submicroscopic malaria standing at 14.5%. Our results revealed that sickle cell trait protects against high parasitaemia and parasite density. As noted all malarial indices were lower in children with HbAS genotype. Children with HbAS genotype had a lower GMPD compared to those with the wild type. But, it was noted that HbAS increases the risk for asymptomatic malaria. These protection seen can be through the enhancement of the acquired and innate immunity, which inhibits parasite proliferation in HbAS carriers. This protection may give a general health advantage for these children. The overall prevalence of anaemia was 59.4% which could be caused by a lot of factors with malaria being a confounder.

6.1.2 RECOMMENDATIONS

- This was a cross-sectional study. A longitudinal study should be done in this district so that we understand the influence of Hb genotype on asymptomatic infection over a period of time.
- There is need to consider Hb genotyping in all infants born in high malaria endemic areas, so that those children who are found to be HbAS are often monitored or examined for malaria and treated if found to be infected.

- Malaria diagnosis should be included as one of the activities during child health week programmes, in areas like Nchelenge.

6.1.3 STUDY LIMITATION

In this study, we used molecular genotyping to measure the effect of infection. The major limitation involved with this method is the inability to detect alleles below the threshold of sensitivity for the given assay. The trend seen is that parasite densities were lower in HbAS than in HbAA children, it is very possible that we underestimated the effect of infection more in HbAS than in HbAA children. This bias was unlikely to have affected our conclusion for a number of reasons:

1. We have shown that no detection of alleles is a function of relative allele proportions more than absolute parasite density (Greenhouse 2006).
2. The assay which we used was sensitive to minority alleles, reliably detecting those present at $\geq$2%.
3. Most importantly, parasite densities were lower in young HbAS than in HbAA children but differences between these groups reduced with increasing age. Therefore, any systematic bias in our finding of increasing protection against the establishment of patent parasitaemia with age in HbAS children would have likely been toward the null hypothesis.

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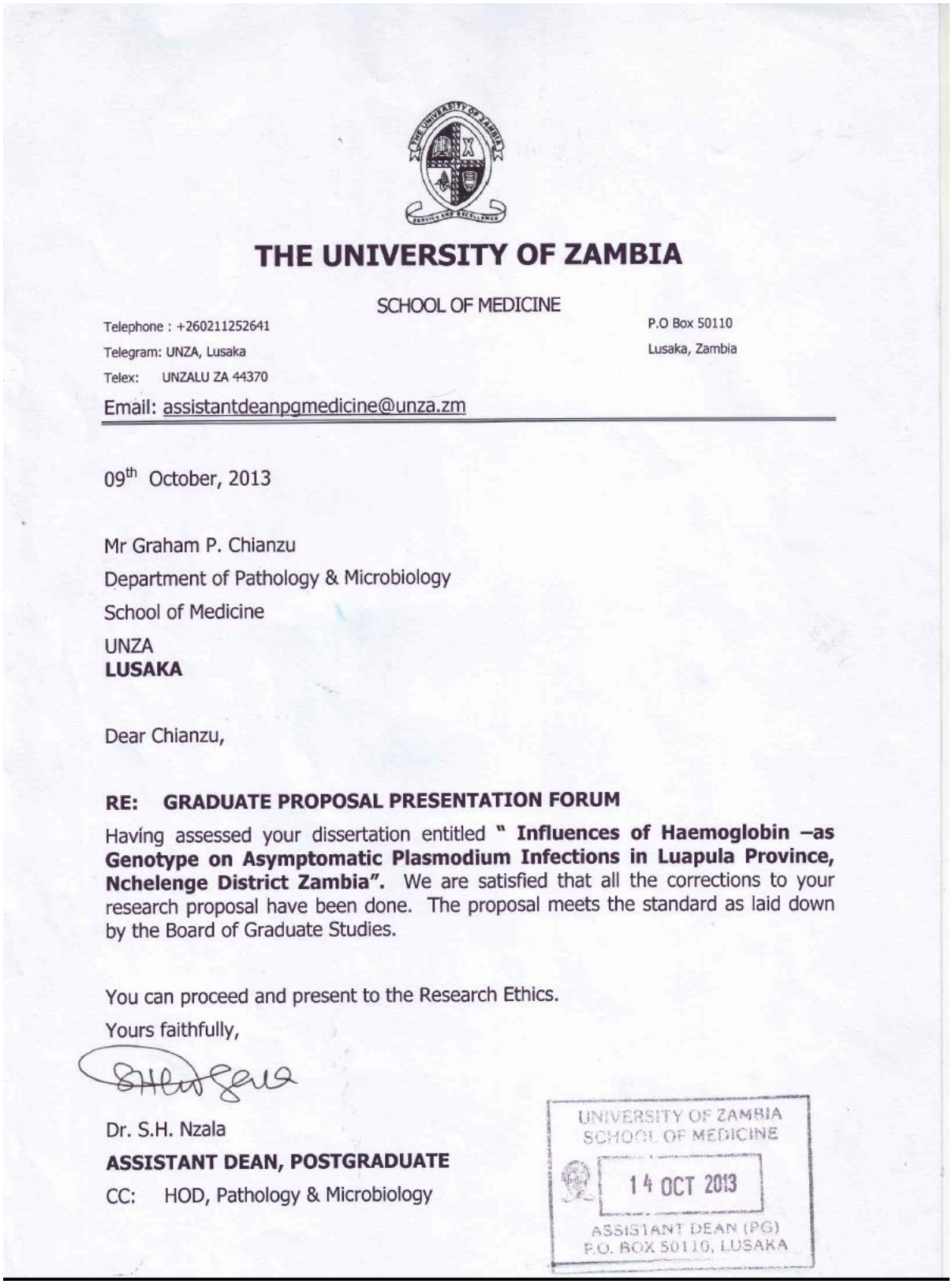
APPENDICES

Appendix 1 Letter of approval of research proposal



THE UNIVERSITY OF ZAMBIA

SCHOOL OF MEDICINE

Telephone : +260211252641
Telegram: UNZA, Lusaka
Telex: UNZALU ZA 44370
Email: assistantdeanpgmedicine@unza.zm

P.O Box 50110
Lusaka, Zambia

09th October, 2013

Mr Graham P. Chianzu
Department of Pathology & Microbiology
School of Medicine
UNZA
LUSAKA

Dear Chianzu,

RE: GRADUATE PROPOSAL PRESENTATION FORUM

Having assessed your dissertation entitled " **Influences of Haemoglobin –as Genotype on Asymptomatic Plasmodium Infections in Luapula Province, Nchelenge District Zambia".** We are satisfied that all the corrections to your research proposal have been done. The proposal meets the standard as laid down by the Board of Graduate Studies.

You can proceed and present to the Research Ethics.

Yours faithfully,

Dr. S.H. Nzala
ASSISTANT DEAN, POSTGRADUATE
CC: HOD, Pathology & Microbiology

UNIVERSITY OF ZAMBIA
SCHOOL OF MEDICINE
14 OCT 2013
ASSISTANT DEAN (PG)
P.O. BOX 50110, LUSAKA

Appendix 2 Letter of Ethical Approval Ethics Committee

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
E-mail: unzarec@unza.zm

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

Assurance No. FWA00000338
IRB00001131 of IORG0000774

4th February, 2014.

Our Ref: 003-11-13.

Mr. Graham. P. Chianzu,
Tropical Disease Research Centre,
Department of Biomedical Sciences,
Haematology Unit,
P.O Box 71769,
Ndola.

Dear Mr. Chianzu,

RE: RESUBMITTED RESEARCH PROPOSAL: **"INFLUENCES OF HAEMOGLOBIN-AS GENOTYPE ON ASYMPTOMATIC INFECTIONS IN LUAPULA PROVINCE, NCHELENGE DISTRICT, ZAMBIA" (REF. No. 003-11-13)**

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 30th January, 2014. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. J.C. Munthali
CHAIRPERSON

Date of approval: 4th February, 2014. **Date of expiry:** 3rd February, 2015.

Appendix 3 Letter of request to use samples from TDRC

TROPICAL DISEASES RESEARCH CENTRE

INTERNAL MEMORANDUM

TROPICAL DISEASES RESEARCH CENTRE (TDRC) 25 SEP 2013 BOARD SECRETARY P.O BOX 71769, NDOLA -ZAMBIA

TO : Director

FROM : Graham P. Chianzu

REF : TDRC/Bio/HAEM/GPC/09/13

DATE : 25th September, 2013

SUBJECT : **REQUEST FOR PERMISSION TO USE ICEMR SAMPLES**

Through: Acting Head of Biomedical Sciences

I write to seek for permission to use blood the blood slides & Dry Blood Spots for study participants aged 6 months to 9 years on the ICEMR study. Furthermore I seek permission to use collected data RDT results and the temperature readings. This is to enable me to conduct My Research, which is the **"Partial Fulfilment of the Requirements for the Degree Master of Science in Pathology (Haematology)"**. The DBS will be used for determining the Haemoglobin genotype of the children and submicroscopic parasitaemia.

The nature of the work that I intend to do is best done on samples collected from households, therefore ICEMR specimens are ideal for my work. My proposal is entitled **"INFLUENCES OF HAEMOGLOBIN-AS GENOTYPE ON ASYMPTOMATIC PLASMODIUM INFECTIONS IN LUAPULA PROVINCE, NCHELENGE DISTRICT ZAMBIA".** This study will be funded by Southern Africa ICEMR, an NIH funded project with John Hopkins University Bloomberg School of Public Health being the Prime Recipient and the University Of Zambia School Of Medicine the Subrecepient.

Attached is my Proposal and letter of award for ICEMR Research Scholarship.

I will be gratefully if this matter is taken into consideration.

Thank you,

GRAHAM P. CHIANZU
SDF-HAEMATOLOGY

CC: DR MIKE CHAPONDA- TDRC ICEMR-PI