

Malaria prevalence, management trends and the knock down resistance profile of *Anopheles gambiae* in Mbakong, Cameroon

NI Cheng

25044672

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Promoter: Prof AF Grobler

Co-promoter: Prof WF Mbacham

Assistant promoter: Prof DA Boakye

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DEDICATION

To my parents Ndong Thomas and Marina Sangha,

To my wife Ernestin Chuo, my children: Ipaingh, Toumeghfeziegh, Asaihkeze and Foughfeziegh (born in the course of this work) for their support and sacrifice.

To my grandmother Ngwi Ching.

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ABSTRACT

Malaria remains a scourge in the world. Recent efforts to combat malaria combine chemotherapy and vector control. Cameroon in 2004 adopted WHO 2001 recommendations and changed her malaria treatment guidelines from monotherapy to artemisinin combination therapy (ACT) as the first-line treatment for uncomplicated malaria and scaled up use of pyrethroid treated nets. Here, we report an investigation into the malaria admissions, management trends and knock down resistance of *Anopheles gambiae* s.s. and *An. coluzzii* under the new policy from 2006-2012. Data was collected from hospital registers and analysed using Statistical programmes (SPSS and SAS). Mosquitoes were caught using human landing catches and *kdr* mutations were detected using PCR.

Of the 4,230 febrile patients received from 2006-2012, 29.3% were confirmed malaria positive with males having a slightly higher risk of having malaria than females (OR=1.08, 95% CI 0.94-1.25). Malaria prevalence fluctuated with a major peak in 2006 and a minor peak in 2011. A practically visible and significant association was observed between age group, gender and having a malaria positive result (Pearson $X^2=153.675$, $p<0.00001$, Cramer's $V=0.352$). The age groups most affected were 5-<14 years and 1-<4 years. Using the Cubic model, malaria prevalence revealed a fluctuating but declining trend over time which was statistically significant and practically visible ($R^2=0.638$, $p<0.0001$).

Treatment data demonstrate that from 2006-2012, a total of 2,556 (60.43%) of the prescriptions dispensed to patients were for an anti-malarial, 1,989 (47.02%) for

antibiotics and 1,935(45.74%) were for antipyretics. The anti-malarials prescribed were ACT 1,216 (47.56%), quinine 1,044 (40.83%) or SP 296(11.62%). Of those prescribed an ACT, only 441(36.27%) had a positive malaria result while quinine intake was recorded in 566 (54.21%) patients positive for plasmodium. ACT prescription increased from 23% in 2007 to between 44%-45% in 2008-2009 while quinine prescription dropped from 38% in 2007 to 13% in 2009 ($r=-0.43$, $p>0.05$).

In terms of the *kdr* mutations, 41.18% of analysed specimens were found to carry the L1014F mutation; 20.59% specimens carried the homozygous susceptible genotype, 19.12% the heterozygous and 1.47% specimen carried the homozygous resistant; 20.83% specimens carried the heterozygous genotype, 2.08% the homozygous resistant genotype while 2.70% carried the homozygous susceptible genotype. This was consistent with Hardy Weinburg equilibrium. The frequency of the L1014S mutations was 31% within the sampled population with 31% within *An. coluzzii* and 24% within *An. gambiae*. The frequencies of both the heterozygous and susceptible genotypes were 11% each, while the homozygous genotype was 2% in *An. gambiae* samples. The frequency of the L1014S mutation in *An. coluzzii* was 19% in the homozygous susceptible and 12% in the heterozygous state.

The changes in the treatment guidelines probably resulted in fluctuating but declining malaria admissions from 2006 to 2012. Over diagnoses and over treatment was common. Control efforts need to be stepped up, with specific focus on the vulnerable groups. In Mbakong the *kdr* mutation frequencies are higher in *An. coluzzii* than in *An. gambiae*. This could negatively affect the success registered under the new policy.

Key words: Malaria, prevalence, trends, management, artemisinin combination therapy, artesunate amodiaquine, artemether lumefantrine, quinine, knock down resistance, sulfadoxine pyrimethamine.

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LIST OF ABBREVIATIONS

A	adenosine
AIDS	acquired immune deficiency syndrome
AB	antibiotic
ACT	artemisinin based combination therapy
AFLP	amplified fragment length polymorphism
AL	artemether lumefantrine
ASAQ	artesunate amodiaquine
bp	base pair
C	cytocine
CHW	community health worker (community relay agent)
cDNA	complementary deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycystidine triphosphate
DDT	dichloro-diphenyl-trichloroethane
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DHS	demographic health surveys
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetate
EtOH	ethanol
G	guanosine

HIV	human immune deficiency virus
HLC	human landing catches
H ₂ O	water
IDR	indoor residual spraying
IPTp	intermittent preventive treatment in pregnancy
IGS	intergenic spacer
ITS	internal transcript spacer
Kb	kilobase <i>knock down resistance</i>
Leu	leucine
L1014F	Leucine to phenylalanine mutation at position 1014
L1014S	Leucine to serine mutation at position 1014
LBW	low birth weight
LLIN	long lasting insecticidal nets
M	molar
MoPH	Ministry of Public Health
mtDNA	mitochondrial deoxyribonucleic acid
NIS	National Institute Of Statistics
NMCP	National Malaria Control Programme
OR	odd ratio (odds)
PCR	polymerase chain reaction
pH	hydrogen-ion exponent
Phe	phenylalanine
PNLCP	Programme National de Lute Contre le Paludisme

Q	quinine
RAPD	random amplified polymorphic DNA
RDT	rapid diagnostic test
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
RBM	roll back malaria
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
sddH ₂ O	sterile double distilled water
s.l.	sensus lato
s.s.	sensus stricto
SSCP	single-strand conformation polymorphism
SP	sulfadoxine pyrimethamine
SSA	sub-Saharan Africa
TAE	Tris-Acetate EDTA
TE	Tris-EDTA
T _M (°C)	melting temperature
Tris	2-amino-2-(hydroxymethyl)-1,3 propanediol
Tri HCl	Tris-hydrochloric acid
T	thymine
μl	microlitre
μM	micromolar
WHO	World Health Organisation
X ²	Chi square
+ve	positive

-ve	negative
<	less than
>	greater than
$\leq$	less than or equal to
$\geq$	greater

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CHAPTER ONE

Problem Statement and Objectives

1.1 Introduction

Malaria is a complex disease that varies widely in epidemiology and clinical manifestations and remains the leading cause of death in the world (Bloland, 2001, Titanji et al., 2008). In 2013 malaria was reported to be endemic in a total of 99 countries compared to 106 countries in 2005 (UNICEF, 2005, WHO, 2013) and remains the most devastating disease in modern times and a public health challenge to sub-Saharan Africa (SSA) in particular. It continues to thrive despite a long history of control efforts (Lemma et al., 2011). Compared to other diseases, malaria is one of the most researched diseases in the world and has received serious attention in recent times. However, less than 10% of the research takes place in Africa where the disease burden is highest (Lewison and Srivastava, 2008). Despite tremendous efforts, knowledge on malaria epidemiology, management and control is still lacking at different levels. This picture is complicated by the lack of information on the prevalence at the local level and across different ecological zones, the eco-adaptation and resistance profile of the vectors and the ever evolving nature of the malaria parasite (Coluzzi et al., 2002). Between 1980 through to 2004, there was a threefold increase in malaria related deaths, which could be explained by two reasons: 1) the temporal rise in resistance to monotherapies including chloroquine and 2) co-infection between malaria and HIV, leading to an increase in the malaria cases and related deaths (Trape, 2001). However, from 2004 to 2010 a reported 30% reduction was achieved in malaria related mortality, mainly in Africa (Murray et al., 2012).

Owing to these difficulties and following lessons from the setbacks of the global eradication programme in the 1950s and 1960s, the WHO adopted the approach of providing technical support to the national malaria control programmes (Talisuna et al., 2004). One of such technical support services were the recommendation of 2001 following an upsurge in malaria mortality due to resistance to the monotherapies such as chloroquine and pyrimethamine. This led the WHO to advise governments to change national malaria treatment guidelines from monotherapy to the more efficacious artemisinin combination therapy (ACTs). Also, countries were encouraged to adopt different vector control approaches such as the use of long-lasting insecticidal net (LLIN). Several countries in the sub Sahara African region adopted the recommendation and changed their treatment policy. Cameroon adopted the recommendation in 2004 and started implementation in 2005 (WHO, 2006).

After the adoption of the recommendation of 2001 and in line with the WHO guidelines of 2006, the government undertook a series of steps to improve on malaria case management and vector control which included; i) scaled up ACT use in 2007, ii) published the new national malaria treatment guidelines in 2008, iii) adopted rapid diagnostics tests (RDTs) in 2009, iv) started free treatment for all under 5 children as well as community management of uncomplicated malaria in 2011 and v) carried out a nationwide free distribution of pyrethroid long lasting insecticidal nets (LLINs) and rolled out RDTs to facilities in 2012 (PNLCP, 2011).

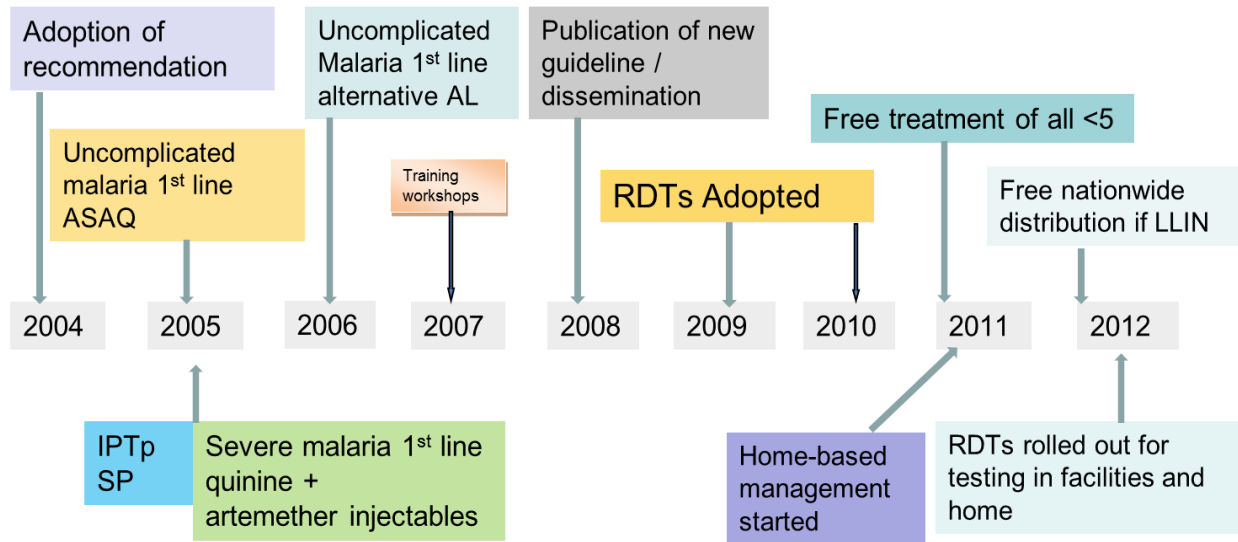


Fig 1.1: Steps taken by the Cameroon government following adoption of WHO 2001 recommendations

Following the implementation of the new malaria treatment guidelines malaria prevalence has reportedly declined from 41% in 2009 to 31% in 2011 and malaria related mortality has reduced from 29% in 2009 to 19% in 2011 while maternal mortality has decreased from 5% in 2010 to 1% in 2011 (NIS, 2012a, NMCP, 2008a, NMCP, 2008b). The world malaria report of 2013 further revealed that between 2000 and 2012, malaria mortality among African children dropped by 54%. This pattern brings to point the need to investigate the prevalence and case management trends over the past seven years of implementation of this policy.

National malaria treatment policies are designed for health professionals who receive patients on a day-to-day basis to guide and facilitate diagnosis, case management and patient follow-up (NMCP, 2008a, Ahir and Bala, 2012). Following the putting in place of the policy it is important to monitor and evaluate the

implementation steps at given points in time. This could help point out the need for adjustments during the implementation process as well as improve performance or scale-up of components of interventions under the policy. A few studies have been conducted in Cameroon to evaluate the state of implementation of the new malaria treatment policy as well as the knowledge and practice of health workers in relation to adherence to test results in malaria case management following the new treatment guidelines (Mangham et al., 2012, Sayang et al., 2009a, Sayang et al., 2009b). These studies revealed that the malaria prevalence was 29% and that malaria was largely over-diagnosed and over-treated with ACTs and quinine. Under the new guidelines ACTs are used as first line for uncomplicated malaria while quinine (Q) and artemether injections are reserved for severe malaria and sulfadoxine-pyrimethamine (SP) for intermittent preventive treatment during pregnancy (NMCP, 2008a). The implementation of the new treatment guidelines has been ongoing from 2005 till date. No study has investigated the pattern of malaria prevalence as well as malaria case management trends over these seven years in Cameroon.

Apart from chemotherapy, vector control has also been instituted and has been ongoing in the past fifteen years (Etang et al., 2004, Etang et al., 2003). This has been scaled-up under the new malaria treatment and control policy (NIS, 2012b). Vector control is an important component of the malaria control policy in Cameroon. Information from vector control efforts helps the National Malaria Control Programme (NMCP) to determine which types of vectors are transmitting malaria in each eco-type, the level of resistance in a particular vector population as

well as determine the type of insecticides to be used at a given time. Molecular markers have been used to detect the level of resistance in some insect species (Miyazaki et al., 1996). One of the resistance markers in the molecular species of *Anopheles gambiae* and *An. coluzzii* that could be assessed to evaluate the extent of resistance spread is the knock down resistance (*kdr*) allele mutation (Touré et al., 1998). Developing a full picture of the distribution of *kdr* allele mutation and associated resistance could help boost control interventions.

Pyrethroid-impregnated bed nets are a central component of the World Health Organization's Global Strategy for Malaria Control (WHO, 1993). They hold particular promise in Africa where 80–90% of the world's malaria cases occur, but there are fears that the emergence of pyrethroid resistance in the *Anopheles* vectors may reduce the effectiveness of this control measure (Ranson et al., 2011) as *kdr* allele mutation confers resistance not only to pyrethroids but also to dichloro-diphenyl-trichloroethane (DDT). Malaria vector control activities under the NMCP in Cameroon focus mainly on the use of insecticide treated bed nets and indoor residual spraying (Bigoga et al., 2007). A rise in resistance to the pyrethroid insecticides due to widespread *kdr* allele mutations that are presently used could jeopardize any success recorded as a result of use of the very efficacious chemotherapies such as the ACTs. Therefore, it is important that in assessing the impact of the recent malaria treatment policy, the extent of resistance of pyrethroid and DDT based insecticides should also be assessed using molecular markers such as *kdr* allele mutations. This highlight the reasons why in addition to studying

the prevalence and management trends of malaria the *kdr* profile of *An. gambiae* and *An. coluzzii* in Mbakong is also studied.

Resistance to insecticides in the malaria vector can be the result of an alternative point mutation at position 1014 in the voltage-gated sodium channel resulting in the leucine-phenylalanine (L1014F) or a leucine-serine (L1014S) substitution termed the knock down resistant alleles or due to the over production of the detoxifying enzymes e.g. glutathione S-transferase, mono-oxygenase or esterase (Beaty and Marquardt, 1996, Santolamazza et al., 2008). Extensive work has been conducted on insecticide resistance in Cameroon (Bigoga et al., 2007, Chouaïbou et al., 2008, Etang et al., 2003, Reimer et al., 2008). But no study till date has been conducted to assess the *kdr* genotype status of the *An. gambiae* and *An. coluzzii* in Mbakong in the North West Region. This work is therefore, intended to throw light on the extent of the spread of the *kdr* mutations in this area and provide baseline data for vector control planners.

1.2 Motivation

Earlier attempts to control malaria in the world were successful but in the end failed for the most part due to the development of resistance by both the parasite to the anti-malarials and the vector to the insecticide used (Wongsrichanalai et al., 2002, Talisuna et al., 2004). This was due to lack of a necessary infrastructure and skills at the national level and proper monitoring of interventions under the programmes. Continuous monitoring in the course of implementation of a health policy could identify early warning signs that resistance to either the drugs or insecticide is

developing. At present there are suggestions that the global malaria prevalence is reducing. However, parallel opinions suggest that figures reported by the WHO are lower than the actual prevalence levels (Murray et al., 2012). If malaria prevalence is reducing, then the prevalence of malaria or febrile illnesses should decline and consequently the global burden should reduce. And in an event of low malaria prevalence, if the global burden of febrile illnesses is still high, it implies some other diseases are associated with malaria that are not being treated. For the burden of malaria to effectively reduce, the low prevalence of the disease should be matched by a corresponding decrease in the vector transmission rate. This could be achieved if the tools adopted for vector control such as pyrethroid insecticides are effective and efficient. However, this is only possible if there is little or no resistance to the insecticides in use. Adoption of closer monitoring schemes could help identify early warning signs to resistance development. One way to monitor the success or failure of a policy is to assess indicators such as prevalence trends, case management trends with the drugs in use, presence of resistance markers in both parasite and vector and the transmission capacity of the vector over a given time when the policy is in force. The case in our study is the implementation of the new malaria treatment guidelines in Cameroon following the WHO treatment guidelines of 2001 within the time frame 2006-2012. The study intends to investigate the trends in prevalence and management of malaria in the Mbakong Health Centre and the knock down resistance profile of *An. gambiae* and *An. coluzzii* in this area following changes in national treatment policy. Some studies have evaluated the policy within a few months using exit polls (Mangham

et al., 2012) or structured interviews (Sayang et al., 2009a, Sayang et al., 2009b) but none has done so using hospital based data over the seven years as well as assessing the *kdr* profile of *An. gambiae* s.s. and *An. coluzzii* in the Mbakong Health area.

It is worth noting that the M and S molecular forms of *Anopheles gambiae* s.s. have been given full species status. The designation “form” (*sensus* Mayr et al., 1953) was ambiguous but it was used to denote units of uncertain taxonomic status. However, in 2013 the molecular forms were given species status. In this light, the M form became *Anopheles coluzzii* (Coetzee & Wilkerson **sp. n.**) and the S form retained the nominotypical name *Anopheles gambiae* (Giles) (Coetzee et al., 2013). In this study we use *An. gambiae* s.s. molecular species to refer to the former *An. gambiae* s.s. (*M and S sibling speices*)

The study site is located along the Mezam and Menchum Rivers near the Menchum fall that has been earmarked for the generation of hydro-electricity. An in-depth understanding of the type of *kdr* mutations and distribution as well as the holistic malaria picture in this area is not only important to the local population, but also to the NMCP to enable it to improve its planning strategy for scaling up of malaria control programmes. This is of critical importance to the health of the non local workers on the dam or tourists visiting the Menchum fall. The results of this study would serve as a baseline for entomological studies in this area.

1.3. Objectives

1.3.1. Aims:

To characterise malaria prevalence, case management trends and the knock down resistance profile of *An. gambiae* Giles and *An. coluzzii* Coetzee & Wilkerson **sp. n.** in Mbakong, Cameroon following changes in malaria treatment policy 2006-2012. This period was specifically chosen as it follows changes in the malaria treatment policy.

1.3.2. Specific objectives

1. Determine the malaria prevalence trends among febrile patients seeking treatment in Mbakong between 2006-2012 following changes in treatment policy.
2. Evaluate malaria case management trends under the new malaria treatment policy from 2006-2012.
3. Determine the *kdr* profile of the *An. gambiae* and *An. coluzzii* using the polymerase chain reaction (PCR). This would demonstrate the extent of spread of the knock down resistance markers in Mbakong populations of these malaria vectors.

The present study is conducted under the framework of collaboration between the Catholic University of Cameroon, Bamenda and the North-West University (Potchefstroom), South Africa. The data was collected from Cameroon and analysed at the North-West University.

The results of the analyses of the hospital data and the molecular work are presented in article format.

This thesis consists of three manuscripts

- Article 1 (Chapter 3): Published by the Malaria Journal August 22, 2014
- Article 2 (Chapter 4): Published by the Acta Tropica Journal June 18, 2015
- Manuscript 3 (Chapter 5): Target Journal: Acta Tropica (To be submitted)

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CHAPTER TWO

LITERATURE REVIEW

2.1 Discovery of the malaria parasite and vector

Malaria is one of the oldest diseases in human history and the leading disease-based cause of death in the world today. Man, the mosquito vector and the *Plasmodium* parasite seem to share certain evolutionary history (Coluzzi et al., 2002). The human-malaria relationship is as old as the human-agricultural-evolution, about 10,000 years ago when malaria started having a major impact on human survival (Hempelmann et al., 2009). Attempts to describe the disease began more than 4,000 years ago as evident from the Hippocratic to Osler eras (Cunha and Cunha, 2008)). The 19th century marked a turning point in malaria history as the malaria parasite, the vector and mode of transmission were identified (CDC, 2008). This paved the way for targeted control strategies and monitoring. In 1880 Alphonse Laveran discovered the presence of malaria parasites in blood. Five year later (1885), Camillo Golgi demonstrated that there were different types of malaria (CDC, 2008, Lewison and Srivastava, 2008). In 1897 Ronald Ross demonstrated that the vector responsible for human malaria transmission was the mosquito (CDC, 2008, Lewison and Srivastava, 2008). Between 1898 and 1899, Giovanni Batista Grassi further demonstrated that the mosquito responsible for human malaria transmission was *Anopheles* (CDC, 2008). In 1902 Giles specifically identified the malaria vector and named it *Anopheles gambiae* (Giles) *sensu stricto*. By the 1960s, it became very clear that there were different species of the *An. gambiae*, a salt loving type (Gebert, 1936), an animal shelter loving type and a zoophilic type (Holstein, 1952, Tonking and Gebert, 1947, Okoye, 2005) in Mauritius. This was backed by similar reports by Bruce-Chwatt, (Bruce-Chwatt, 1974) in the neighbouring island of Reunion (Coluzzi et al., 1979a). Owing to these discoveries different attempts have been made to eradicate or bring malaria under control.

2.2 Evolution in Malaria control

Malaria control has evolved over the last century through different phases. This includes the use of a sanitation approach, preventing man-vector contact using nets and use of chemicals against the parasite and the vector. The history of malaria control dates back to the days of the Romans where malaria control was sanitation based through the draining of stagnant water (Cunha and Cunha, 2008). Following the discovery of Ross and Batista Grassi, efforts were made to control malaria – Ronald Ross proposed the sanitation drive, which was successful in Cuba in 1901 and during the construction of the Panama canal in 1905-1910, in Greece in 1906, and in Mauritius in 1907-1908, while Batista Grassi proposed the use of tight nettings to prevent man-vector contact (CDC, 2008), which forms the bases for today's vector control using insecticide treated nets.

By the 1970s it had become clear that *Anopheles gambiae* consisted of a complex and there was growing concern that proper identification of members of this complex could help pave the way to solving the problem of malaria control (Coluzzi et al., 1977). Till date, the quest for the proper identification of the malaria vector has not ceased. This huddle is further compounded by the lack of a proper understanding of the resistance profile of the different vectors across different ecological regions.

Apart from vector control, the first step toward the use of chemotherapy was the use of quinine in treating malaria. Quinine is obtained from the bark of the Cinchona tree. Historical sources reveal that it was first discovered in 1632 and remained the main antimalarial agent until the 19th century, when primaquine and quinacrine were introduced and later the introduction of chloroquine in 1934. The latter became the drug of choice for malaria case management in 1946 (Achan et al., 2011, Talisuna et al., 2004, Wongsrichanalai et al., 2002). These methods of control were however not used on a large scale. In 1955 the World Health Organization

embarked on a global eradication programme, which included the use of antimalarial drugs such as chloroquine and pyrimethamine and insecticides such as DDT for indoor residual spraying. This step was based on the premise that the parasite lack animal reservoirs and effective agents that could block transmission and bring about a radical cure were available (Talisuna *et al.*, 2004). Though this strategy was initially successful in some parts of Asia such as India, it was short lived due to overwhelming challenges.

2.3 Challenges encountered by the Global Eradication Programme and the way forward

Many of the national programs of the countries that implemented this programme did not have the adequate epidemiological skills, the knowledge, the needed administrative organization and the infrastructure to continuously monitor the progress of the activities (Talisuna *et al.*, 2004). Some of the factors that contributed to the low success rate of the programme include among others development of resistance to drugs and insecticides, mass population movements due to wars, lack of populations' ownership of the programme, exophilic mosquitoes that do not rest long enough to take up the lethal dose of the insecticide were targeted and lack of funding caused the campaign to be abandoned. Furthermore, not all regions of the world were involved. For instance, sub-Saharan Africa was not included in the campaign (Curtis and Lines, 1985, Talisuna *et al.*, 2004, WHO, 1993b, WHO, 1993a). This suggests that even if the programme was successful, malaria would have remained a problem to Africa and eventually the world.

Following the failure of the programme the WHO shifted its focus from malaria eradication to control (CDC, 2008). Different efforts have since then been made to improve the control strategies through policies that encourage the use of guidelines that are developed from evidence based research. In 1992 the Amsterdam Ministerial Conference on Malaria Control Strategies with the goal to reduce morbidity and mortality, adopted as one of the key recommendations the provision of early diagnosis and prompt access to treatment with anti-

malarials – chloroquine (CQ) and sulfadoxine pyrimethamine (SP) (Talisuna *et al.*, 2004). Also the international community set targets that were geared toward rolling back malaria by 50% by 2010 and by 75% by 2015. This constituted part of the World Health Assembly 58 resolutions of 2005 and the recommendations of the Abuja Declaration of 2006 (WHO, 2006a). Research and surveillance through the use of indicators such as prevalence rates, transmission level in a given zone and capacity of the vector, resistance to drugs by parasite and the resistance to insecticides by the vectors as well as proper identification of the vector are considered as crucial tools for informing policy on malaria control strategies (Wondji *et al.*, 2005). Despite the failure registered in the malaria eradication programme in some areas, the campaign was successful in America and Europe. Through continuous efforts and strategy improvement towards malaria control and eventual eradication, the WHO (2011) recently certified four countries to have eliminated malaria: United Arab Emirates (2007), Morocco (2010), Turkmenistan (2010) and Armenia (2011) (WHO, 2011). This definitely suggests that the malaria control approach is making progress and the malaria epidemiology map is changing over time. In the following sections we will zoom into the evolution of malaria control efforts, transmission and the vector as well as malaria policies to control malaria in the world, Africa and Cameroon in particular.

2.4 Epidemiology of malaria

2.4.1 Global burden and distribution of malaria

It remains the most devastating disease in modern times and a public health challenge worldwide and sub-Saharan Africa (SSA) in particular despite a long history of control efforts (Lemma *et al.*, 2011). In 2013, WHO reported that malaria was endemic in a total of 99 countries in the world compared to 106 countries in 2005 (UNICEF, 2005, WHO, 2013a). The present burden of malaria may be compounded by HIV, civil unrest, food shortage and drought (Chin, 2000). Incidentally, 80% of malaria related deaths occur in 14 of the endemic countries

with the Democratic Republic of Congo and Nigeria contributing more than 40% of the world mortality burden (WHO, 2013). Malaria alone contributes 2.3% of the world's disease burden with 90% occurring in sub-Saharan Africa (Lewison and Srivastava, 2008). By 2002, it was reported that an estimated 0.7- 2.7 million people died each year from malaria with more than 75% being African children (Wongsrichanalai et al., 2002). In 2006 about 250 million episodes of malaria were registered the world over with close to a million deaths, mostly under five children (Lemma et al., 2011, WHO, 1975). Four years on, in 2010, 216 million episodes of malaria and an estimated 655,000 deaths were registered of which 81% of episodes and 91% of deaths occurred in Africa (WHO, 2011). The world malaria report of 2013 further revealed that between 2000 and 2012 the malaria mortality among Africa children dropped by 54% (WHO, 2013b). Though some improvements have been achieved especially following the WHO guidelines of 2001 both at the level of reducing morbidity and mortality, malaria consistently remains the leading cause of morbidity and mortality in the sub-Saharan African region (Otten et al., 2009, Lemma et al., 2011, Murray et al., 2012).

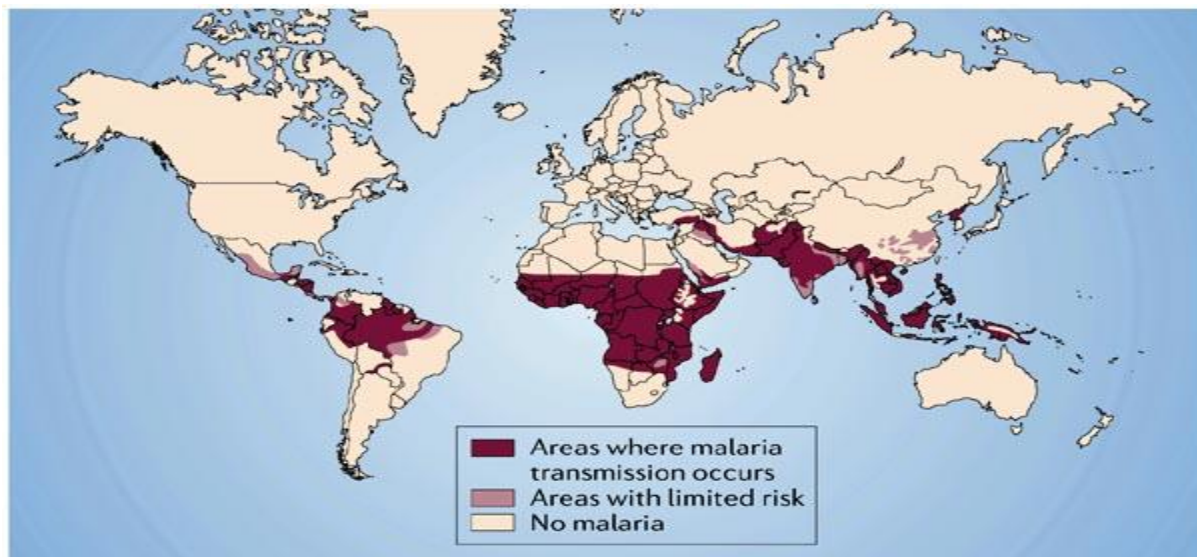


Figure 2.1: World Malaria Burden (Adapted from Bell et al., 2006: (4) 7-20, with permission from Nature Publishing Group)

A recent systematic analysis by Murray and colleagues on global malaria mortality between 1980 and 2010 revealed that globally, malaria deaths increased from 995,000 in 1980 to a peak of 1,817,000 in 2004, and decreased to 1,238,000 in 2010. Though globally, death due to malaria is seen to have increased, the actual increase took place only in Africa as malaria mortality increased from 493,000 in 1980 to 1,613,000 in 2004, and decreased by about 30% to 1,133,000 in 2010 (Murray et al., 2012). Outside of Africa, malaria deaths have steadily decreased from 502,000 in 1980 to 104,000 in 2010. The importance of this finding is that more deaths occurred worldwide in 2010: 435,000 and 89,000 in and outside Africa respectively in individuals aged 5 years and older than was suggested by previous studies and WHO estimates (WHO, 2011). The three times rise in mortality between 1980 through 2004 could be explained by two reasons: 1) the temporal rise in chloroquine resistance and 2) co-infection between malaria and HIV leading to an increase in the malaria cases and related deaths (Trape, 2001).

Malaria accounts for approximately 22% of all childhood deaths. This group and pregnant women have remained the most vulnerable and have the highest risk of acquiring malaria (WHO, 2011). Pregnancy related malaria is detrimental to the health of both mother and child with consequences such as abortion, stillbirths and low birth weight (Lindsay et al., 2000). Low birth weight accounts for a fatality rate of 37.5% (Worral et al., 2007). Reports further suggest that between 75,000 and 200,000 infant annual deaths are linked to pregnancy associated malaria (Shulman and Dorman, 2003, Steketee et al., 2001) and more than 50 million pregnant women are at risk of malaria (RBM, 2008).

The burden of the disease is most evident mainly in poorer tropical areas of Africa, Asia and Latin America inhabiting about 40% of the world population (WHO, 1999). In Africa, the sub-Saharan African region is the hardest hit bearing about 60% of the clinical cases and 80-85% of the deaths (Lemma et al., 2011, Stratton et al., 2008, UNICEF, 2005). Historically, only

HIV/AIDS in the 20th century seemed to have rivaled malaria in terms of the number of people killed annually. In the sub-Sahara African regions the high prevalence of HIV further compounds the malaria problem. Due to the high cost of malaria case management, infrastructure and inputs required for control efforts both at the level of the individual and state, malaria places an economic burden on limited resources rendering them poorer. The higher the malaria morbidity and mortality, the lower the contribution to economic returns to the family and state and the poorer they become (Stratton et al., 2008). Thus, malaria and poverty are involved in a vicious cycle. This cycle is most evident when we take a closer look at individual countries and in our case we will be focusing on the situation in Cameroon.

2.4.2 Malaria prevalence in Cameroon

Cameroon, located in the heart of Central Africa sharing the Forest, Savannah, Sahelian and desert ecotypes with different microclimates, is a hot spot of genetic diversity both at the level of the malaria vector (Etang et al., 2009b) and parasite. Malaria remains a major public health problem in Cameroon (Titanji et al., 2001). It is endemic with varying levels of prevalence and transmission across the different ecological zones or niches (Bigoga et al., 2007b). Recent studies in Southern Cameroon revealed a prevalence of 29% and in children <5 years it stands at 30% while in the rural areas prevalence is estimated to be 37% (Mangham et al., 2012, NIS, 2012).

The epidemiologic profile of malaria in Cameroon is three fold: i) An endemic and perennial transmission zone covering the southern equatorial forest, coastal and western plateau with 7-12 months of rainfall, ii) an endemic and seasonal transmission zone in the Adamawa plateau and savannah forest with 4-6 months of rainfall and iii) an epidemic and strongly seasonal zone covering the Sudano-sahelian region with seasonal transmission of 1-3 months (Ngum et al., 2010, Bigoga et al., 2012). Incidentally, it is the major cause of morbidity and mortality among

the most vulnerable groups: children under five (18%), pregnant women (5%), people living with HIV/AIDS (5.5%), and the poor (40%), which constitute 2/3 of the total population estimated at 19 million (Bigoga et al., 2007b). Over 900,000 clinical cases of malaria occur yearly accounting for 50 to 56% of morbidity among children under age five, 40 to 45% of medical consultations, and 30 to 47% of hospitalizations (NIS, 2012; NMCP, 2008). Also, it is responsible for 49% of prenatal consultations and 59% of hospitalizations during pregnancy leading to abortions, premature labour and deliveries and low birth weight (LBW), thereby exposing babies and mothers to the risk of early deaths (Ngum et al., 2010).

Despite efforts by the NMCP to curb the disease burden, the prevalence of febrile illnesses attributed to malaria is seemingly on the increase (Mangham et al., 2012). Previous studies have attributed this to the increasing spread of drug resistance in the parasite, insecticide resistance in the vectors, inadequate and inconsistent allocation of resources for control (Etang et al., 2003, Quakyi et al., 2000), and the presence of very efficient mosquito vectors of *Plasmodium falciparum* (Bigoga et al., 2007b, Etang et al., 2006, Manga et al., 1993). This was compounded by a poor monitoring and evaluation system for control interventions in the past, which is now increasingly a component of most control programmes.

2.4.3 Renewed commitment to reducing the malaria burden

Following the 2005 global commitment to reducing the burden of malaria by the international community by at least 50% by 2010, it was recommended that countries undertaking malaria control interventions should attain at least a >80% coverage rate. The interventions included long-lasting insecticide treated nets, indoor residual spraying (IRB), intermittent preventive treatment of pregnant women (IPT) and treatment with effective anti-malarials especially artemisinin-based combination therapy (ACTs) (Otten et al., 2009, RBM, 2005).

In many African countries, there is increasing evidence of a significant reduction in malaria transmission, morbidity and mortality where malaria control interventions have been implemented (Trape et al., 2011, Otten et al., 2009, WHO, 2006a). Data reporting a decline in malaria although not always spatially harmonious, clearly illustrate a sharp decline in the malaria prevalence rate in sub-Saharan Africa in children aged two to ten years – from 37% in the years 1985–1999 to 17% in 2000–2007 (Guerra et al., 2008). In a recent study Otten *et al*, reported that the number of in-patients malaria cases and deaths in the under five year children dropped by 55% and 67% respectively in Rwanda and by 73% and 62% in Ethiopia between 2001 and 2007 following a combination of scaled up interventions (Otten et al., 2009). This decrease implies that many of the areas previously defined as “high stable malaria transmission” have changed, or will soon change, into “moderate to low transmission areas” or even to epidemic areas (D'Acremont et al., 2009, Otten et al., 2009) – thus reducing the likelihood that a fever episode will be associated with malaria (Wang et al., 2006). Therefore, seeking confirmation for malaria for given febrile episode need not just be part of a policy option for health workers or care givers but it is important that the health worker be ready to accept the result, act on it as well as request further investigation where necessary. This is due to the fact that increasingly, not all causes of fever are linked to malaria as has been traditionally believed (Roucher et al., 2012).

In Cameroon, according to the NMCP report of 2011, malaria accounted for 31% of hospital consultations in 2010 compared to 38% in 2009 and 41% in 2008. Malaria related mortality in the general population dropped from 29% in 2008 through 24% in 2009 to 19% in 2011 while mortality among pregnant women drop from 5% in 2010 to 1% in 2011 (Minsanté, 2009, NIS, 2012, PNLCP, 2011). The observe trend could only be sustained if there is continued commitment to use all available resources that assist the proper diagnosis and facilitate appropriate case management.

2.5 Malaria diagnosis and case management

2.5.1 Clinically/confirmed malaria diagnosis

Malaria is typically diagnosed clinically using signs and symptoms or with the aid of tools such as microscopy or rapid diagnostic kits. As mentioned earlier, malaria has recently been reported to be declining in prevalence across different parts of Africa. If malaria prevalence is reducing, it should be expected that the confirmed malaria related admissions or fevers associated with malaria should decline. In most malaria endemic settings in Africa including Cameroon, clinical diagnosis of malaria is a routine practice. This obviously results in over-diagnosis and over-treatment even when testing services such as microscopy are in place (Mangham et al., 2012, Mubi et al., 2011, Reyburn et al., 2007).

To solve the problem of widespread over-diagnosis and over-treatment of malaria, the WHO in 2010 recommended new malaria treatment guidelines (Chandler et al., 2012). The recent WHO guidelines recommended parasitological confirmation of suspected malaria cases in all patients before treatment, where testing facilities are available (WHO, 2010). If malaria prevalence is reported to be declining, the hazard of misdiagnosis becomes significant. This is because when presumptively treating for malaria, health workers are less likely to seek for another treatable cause of fever, which may only be realized at an advanced stage. This therefore, results in higher morbidity and mortality in non-malaria cases due to delay in the onset of appropriate treatment, and indeed, case fatality rates have been reported to be higher among non-malaria fevers compared to malaria fevers (Reyburn et al., 2004). This argument would be firmly supported in a non endemic zone for malaria. However, in a malaria endemic zone, the residual parasitaemia turns out to be higher in patients with partial immunity. Over dependence on the malaria test positive results as a cause of the fevers then minimise the actual cause of non malaria fevers (Graz et al., 2011).

2.5.2 Asymptomatic malaria in a high transmission zone.

Due to the high prevalence of asymptomatic malaria infections and the non-specific signs and symptoms of the disease, the diagnosis of clinical malaria presents serious drawbacks in malaria endemic areas. This is critical for making first-line treatment decisions and for accurate evaluation of any intervention aimed at malaria morbidity reduction (Roucher et al., 2012).

Although early recognition of symptoms and signs perceived as malaria are important for effective case management, the predictive values of these signs alone are often poor in stable malaria transmission areas (Ndyomugenyi et al., 2007, Oyibo, 2012, Oladosu and Oyibo, 2013). Case management thus depends on early identification and interpretation of symptoms and signs such as a malaria episode, in addition to the clinical skills of a healthcare worker as there are often no resources for laboratory diagnosis in most malarious endemic areas (Van den Ende et al., 1998, Font et al., 2001). Perceived fever is the sign most health workers use to diagnose clinical malaria but studies in areas of intense transmission have found reported fever or a history of fever to be an unreliable indicator of clinical malaria. Ndyomugenyi *et al* (2007) found that in a low transmission area in Uganda, the symptoms such as headache, vomiting, and rigors were significantly associated with parasitaemia while dizziness and cough had negative association with parasitaemia, indicating that the specificity and positive predictive value of clinical signs and symptoms were generally low (Ndyomugenyi et al., 2007, Nsagha et al., 2011).

In a high transmission area the level of parasitaemia in asymptomatic malaria infection could be higher than in low transmission area. In such an area when a patient has fever or other malaria symptoms, the presence of *Plasmodium spp* neither reliably confirms malaria as the cause of the fever, nor excludes the possibility of other diseases. This is because the patient may be an asymptomatic carrier and suffers from another disease. It is thus, important to use the local

epidemiologic data alongside the clinical skills of the healthcare giver. However, in low transmission areas high parasitaemia could reliably be linked to a fever (Graz et al., 2011, WHO, 2006b). The above picture further suggests that combining the health worker skills, diagnosing appropriately as well as using diagnostic results as reliable indicators could facilitate case management in malaria and related illnesses.

However, while reports on field observations have stressed the fact that health care workers do not accept or adhere to parasitological test results, the practical situation of the healthcare worker may be very challenging even to malaria experts. Firstly, when the WHO guidelines classified according to level of transmission: high transmission zone, moderate to low or unstable transmission zones (WHO, 2006b), it reflects a classification that should match all the health districts and health areas in the field. Most of the time, this seems not to be the case, and the transmission profile in some areas is not well documented. Some of these ambiguities are at times also translated into the national guidelines. Secondly, the health worker seems to grab onto and manage only with whatever new knowledge they think will best help them in their practice, because it takes time for the health worker to change a particular habit. Yet, the time from when one modification is made in the guidelines to when another modification is introduced seems to be growing ever shorter. The continuous modification of guidelines, especially at short term, creates a gap between the health policy makers and those implementing the policy in the field, most of the time leaving the health system ever-wanting.

2.6 Malaria case management

2.6.1 Treatment of clinical and confirmed malaria

Following the failure of the eradication programme in the 1960s, the alternative strategy adopted by the WHO was malaria control. This approach had and continues to have as a key component

the provision of guidelines that could be adapted to match the local context of each partner country. One such event happened in 2001 when the WHO recommended the use of artemisinin-based combination therapy (ACT) for the treatment of uncomplicated malaria. This was targeted at settings where resistance to conventional treatment such as chloroquine and sulfadoxine pyrimethamine (SP) was reported (WHO, 2006). Owing to the fact that this change in the treatment guideline was due to resistance, the question was how to prevent this from happening to the highly efficacious combination therapy in the near future.

To address the above concerns, intervention studies in different parts of Africa have investigated patterns of malaria treatment and the effect of improving diagnostics (microscopy and introducing RDTs) on treatment outcome (Ansah et al., 2010, Juma and Zurovac, 2011, Mangham et al., 2011, Mubi et al., 2011, Ngasala et al., 2008, Rafael et al., 2006, Sayang et al., 2009). In Tanzania, Mubi et al. (2011) studied the impact of rapid diagnostic test (RDT) aided management of febrile patients as compared to clinical diagnosis alone by community health workers (CHW) for targeted provision of artemisinin-based combination therapy (ACT) for uncomplicated malaria at community level. The results demonstrated that the use of RDTs by CHWs can be safe and effective for targeting ACTs to patients with uncomplicated malaria in sub-Saharan Africa, corroborating an earlier report in Zambia (Yeboah-Antwi et al., 2010). The use of RDTs significantly reduced prescription of ACT by 45% compared to clinical diagnosis, with the largest reduction of treatment (57%) occurring in patients above 15 years (Mubi et al., 2011).

Although appropriate use of ACT is especially important in children, reduced over-use of ACT among adults will be a drug-cost saving besides preventing unnecessary drug exposure. The reduced prescription of ACT as a result of using RDTs could be achieved if health workers

adhere to RDT results. This means that a negative result must be accepted and other causes of the illnesses investigated (Wang et al., 2006), which is achievable through enhanced community health worker training and sensitization that will help community members to pay for the test. Previous findings of poor adherence to negative test results at health facility level have been ascribed to the poor quality of the tests and fear of false negative results (Reyburn et al., 2007). In another randomized controlled trial, Ngasala et al. (2008) investigated the impact of training in combining clinical and microscopy diagnosis against using clinical diagnosis alone on the prescription of anti-malarials for childhood malaria as well as the health outcome at primary healthcare levels. It was found that those with positive tests were less likely to be prescribed antibiotics than those with negative tests (Ngasala et al., 2008). In Ghana a recent report observed that the use of RDTs significantly improved the prescription habits of health workers in health facilities that had no microscopy services but had little effect on facilities that already used microscopy (Ansah et al., 2010).

2.6.2 Cameroon puts the new treatment policy in place

National malaria treatment policies are put in place for health professionals who receive patients on a day-to-day basis to guide and facilitate diagnosis, case management and patient follow up (NMCP, 2008, Ahir and Bala, 2012). Cameroon adopted the 2001 WHO treatment guidelines in 2004 (NMCP, 2008) and following the WHO recommendations, changed her malaria treatment policy from monotherapy to ACTs with artesunate-amodiaquine (ASAQ) as the first-line treatment for uncomplicated malaria, which is subsidised by the government. Following allegations that ASAQ had serious side effects, artemether-lumefantrine (AL) was adopted as an alternative ACT in 2006 which, however, is not subsidized. Quinine (Q) and artemether injectables under this policy are jealously reserved for cases of severe malaria while sulfadoxine-pyrimethamine (SP) for intermittent preventive treatment during pregnancy, followed

by a series of training workshops to acquaint the professionals of the health sector with the new policy. This culminated in the 2008 national malaria treatment guidelines that were republished by the NMCP (Sayang et al., 2009, NMCP, 2008, Mangham et al., 2012) and made available to all health facilities.

2.7 Implementation of the new malaria treatment policy

Effective implementation of the new malaria treatment policy is reported to have started in 2005 with about 50% of the health facilities involved (NMCP, 2008). In the same year, Sayang et al. reported that 13.6% of healthcare professionals knew about the new guidelines and 73.5% supported the use of ASAQ as first line therapy but 38.6% did not recommend it due to the alleged side effects (Sayang et al., 2009). In a clustered randomised control trial (Mangham et al., 2012, Wiseman et al., 2012), it was found that all health facilities had adopted the new policy by 2010. However, widespread deficiencies have equally been reported in the prescribing practices of health workers responsible for treating febrile patients attending public and private facilities in Cameroon. The Demographic Health Survey (DHS) of 2011 demonstrated that though not subsidised, the ACT AL was most often prescribed compared to the highly subsidised ASAQ (NIS, 2012, WHO, 1993b). This was further supported by the report of the NMCP, which revealed that even in public healthcare facilities AL was more frequently prescribed than the subsidised ASAQ (PNLCP, 2011).

Furthermore, the recommended anti-malarials are intended to be put to appropriate use, which is only possible through accepting and using the testing services that were established. In earlier guidelines parasitological confirmation was not emphasized in high transmission areas. However, due to widespread over-use of anti-malarials and unnecessary drug exposure, the WHO changed its guidelines in 2010 and recommended a parasitological confirmation for all

suspected malaria case where testing services are available (Chandler et al., 2012, WHO, 2010). Despite the availability of microscopes for malaria testing in public and private facilities in Cameroon, symptomatic diagnosis of malaria remains routine in more than 50% of consultations (Mangham et al., 2012). Mangham et al. (2011) observed that prescription of anti-malarials to patients with a fever in some health facilities in Cameroon was not based on results of malaria diagnosis. In line with this, 73% of all febrile patients were prescribed or received an antimalarial though malaria was confirmed in only 29% of patients using RDTs. Poor adherence to negative results was equally observed with up to 70% of malaria negative patients being prescribed or receiving an antimalarial, while 51% were presumptively prescribed an ACT. Such practice of poor adherence to test results has also been observed in other parts of Africa (Zurovac et al., 2012, Ansah et al., 2010).

Following the national treatment guidelines, the health worker's clinical decision based on the signs and symptoms is important as upon confirmation of malaria, it determines whether the patient is classified as suffering from uncomplicated or severe malaria. This in turn influences the type of prescriptions given a patient. Though the guidelines do stress that the identification of *Plasmodium* parasites is a conclusive diagnosis for malaria in ≥ 5 year old patients, it further states that the absence of parasites in a blood film does not rule out malaria. The latter introduces a serious lack of coherence in the guidelines as well as opens an opportunity for presumptive diagnosis or poor adherence to the test results, a common practice in some parts of Africa despite the existence of the treatment policies (Ansah et al., 2010, Mangham et al., 2012, Reyburn et al., 2007). Reports underline that when presumptive treatment is practiced the proportion of patients who receive appropriate treatment are reduced (Mbonye et al., 2013).

To further improve the drive for the quality of care offered the population and the appropriate use of ACTs the government in 2009 endorsed RDTs to enhance testing services (Minsanté, 2009) and restarted home based management of malaria through relay agents at the community level by community relay agents. In 2012, RDTs were rolled out to all public facilities and also for the community-based management of uncomplicated malaria (PNLCP, 2011), in line with the WHO treatment guidelines of 2010 (WHO, 2010). Many studies have demonstrated that malaria is highly over-diagnosed as well as over-treated in Tanzania (Reyburn et al., 2007), Angola (Rowe et al., 2009), Kenya (Juma and Zurovac, 2011) and recently in Cameroon (Mangham et al., 2012) where 70% of patients with malaria negative results were reported to be prescribed an ACT.

In an attempt to curb the over-use of anti-malarials,, many interventions have been conducted to evaluate the impact of using RDTs and also to improve providers' knowledge about their prescription habits in line with new policies in Ghana (Ansah et al., 2010), Uganda (Kyabayinze et al., 2010), Kenya (Zurovac et al., 2006, Skarbinski et al., 2009), Tanzania (Ngasala et al., 2008), Zambia (Yeboah-Antwi et al., 2010), Nigeria (Mangham et al., 2011) and Cameroon (Mangham et al., 2012). These studies have shown that between 35-85% of RDT negative patients are still treated with anti-malarials (Chandler et al., 2012). Such studies have been conducted in most instances to evaluate the level of implementation of the national treatment policy. In a baseline study in Cameroon, Mangham et al. (2012) reported an improvement in the use of ACTs and quinine compared to the report by Sayang et al, in 2009 (Mangham et al., 2012, Sayang et al., 2009). A recent study revealed that the implementation of RDTs as well as interventions tailored to enhance the translation of health worker knowledge into prescribing practice greatly reduced the over-prescription of antibiotics and improve quality of patient care (Mbacham et al., 2014).

2.8 The cost impact

Applying the appropriate control strategy has a cost impact on the population and the government. Though some of the anti-malarials are subsidized, patients seem to prefer the unsubsidized alternatives that are most often more expensive (NIS, 2012, PNLCP, 2011). ACTs have been reported to be more expensive than chloroquine and the need for a diagnosis only increases the cost (Mangham-Jefferies et al., 2014, Rafael et al., 2006). The high cost of treatment goes a long way to support the argument that appropriate diagnosis prior to treatment is essential for healthcare provision in an era where malaria transmission is reported to be declining. In addition to the direct cash cost, attention has not been given to the cost due to loss in working days on different members of the family and the pain patients go through as a result of unnecessary treatment. Though presumptive treatment and microscopy have been used for a long time, adoption of RDTs has been shown to limit unnecessary drug use if health workers accept the result. In a recent study, Uzochukwu and colleagues evaluated the cost effectiveness of rapid diagnostic tests, microscopy and the presumptive treatment of malaria based on fever in Nigeria (Uzochukwu et al., 2010). The finding demonstrated that RDTs were more cost-effective over microscopy and presumptive treatment and this tallied with the findings of a previous study by Shillcutt (Shillcutt, 2008). However, chemotherapeutic and diagnostic approaches cannot be used in isolation for the effective control of malaria without the involvement of preventive strategies such as vector control, which is geared toward reducing man-vector contact and consequently reducing transmission.

2.9 Malaria transmission in Cameroon

Malaria transmission in Cameroon vary according to ecotype with the periodicity of high entomological inoculation rates coinciding with periods of increased vector density, biting rates, rainfall and prevalence of malaria parasitaemia in the indigenous population (Bigoga et al.,

2007b). The implementation of vector control strategies that interrupt transmission can only be effective if its planning is based on relevant available information about the vector population structure, their distribution, efficiency in malaria transmission and their insecticide resistance status, especially that of *kdr*.

2.9.1 Malaria vectors and distribution in Cameroon

Malaria vector studies in Cameroon have been scaled up within the past two decades. This has revealed that the vector species, intensity and duration of transmission vary greatly between different ecological zones of the country. In the southern forested region, transmission is mostly perennial; seasonal in the Sudano-savannah and unstable in the northern Sahelian savannah regions (Bigoga et al., 2007b, Fontenille and Simard, 2004, Wanji et al., 2003, Wondji et al., 2005, Manga et al., 1993, Antonio-nkondjio et al., 2006). Forty-eight different species of *Anopheles species* have been described in Cameroon and 14 have been found to be implicated in malaria transmission (Antonio-nkondjio et al., 2006). There are five major malaria vectors; *Anopheles gambiae* Giles, *An. coluzzii* Coetzee and Wilkerson **sp. n.**, *An. Arabiensis* Patton, *An. funestus* Giles, *An. nili* Evans, and *An. moucheti* Theobald, which contribute 95% to the observed malaria transmission in Cameroon (Fontenille and Simard, 2004, Achan et al., 2011). Nine secondary vectors contribute the remaining 5%. The latter include *An. paludis* (Theobald), *An. hancocki* (Edwards), *An. ovengensis* (Awono-Ambene et al.), *An. carnevalei* (Brunhes et al.), *An. coustani* (Laveran), *An. marshalli* (Theobald), *An. pharoensis* (Theobald), *An. wellcomei* (Theobald) and *An. ziemanni* (Grunberg) (Antonio-nkondjio et al., 2006, Antonio-Nkondjio et al., 2005, Bigoga et al., 2007a, Fontenille and Simard, 2004, Mouchet, 2004, Wanji et al., 2003).

In terms of malaria transmission, members of *An. gambiae* and *An. funestus* are the most potent malaria vectors and are widely distributed in Cameroon from the southern forested to the northern rice farming fields. *An. moucheti*, *An. nili*, *An. hancocki*, and *An. paludis* are prevalent

mostly in the forest area though they have also been reported in the dry savannah. In the forest region, the main malaria vectors *An. moucheti* and *An. nili* are riverine. While *An. moucheti* colonises areas along fast flowing rivers, *An. nili* colonises areas along slow flowing rivers (Bigoga et al., 2007b, Chouaïbou et al., 2008, Etang et al., 2003, Nwane et al., 2011, Quakyi et al., 2000, Wanji et al., 2003, Wondji et al., 2002, Ndong et al., 2014, Antonio-nkondjio et al., 2006). The secondary vectors are not distributed nationwide but nevertheless play a crucial role in malaria transmission at the bio-ecological locality where they occur (Antonio-nkondjio et al., 2006, WHO, 2013b, Wanji et al., 2003, RBM, 2008). *Anopheles nili*, *An. moucheti*, and *An. hancocki* are known to contribute to transmission in the Mount Cameroon region (Wanji et al., 2003), *An. ovengensis* and *An. pharoensis* contribute about 34% and 8.5% respectively, to malaria transmission in Tibati (Antonio-nkondjio et al., 2006) while in Nieme in the Southern plantations *An. ziemanni*, *An. paludis* and *An. smithii* (Theobald) are known to contribute to malaria transmission (Bigoga et al., 2012).

Of the seven members of the *An. gambiae* s.l. complex, four have been reported in Cameroon: *An. gambiae* s.s. and *An. coluzzii*, *An. melas* and *An. arabiensis* (Bigoga et al., 2007a, Etang et al., 2003, Manga et al., 1993, Wanji et al., 2003, Wondji et al., 2002). In a recent study in South Western Cameroon, Bigoga and colleagues demonstrated that *An. gambiae* and *An. coluzzii* have the highest infective bites per night (Bigoga et al., 2012) while Wanji and colleagues have observed an entomological inoculation rate of 161 infective bites per year at the Mount Cameroon region of South West Cameroon (Wanji et al., 2003).

2.9.2 The *Anopheles gambiae* complex

Members of the *An. gambiae* complex have been reported in all the ecological zones in Cameroon. In terms of distribution *An. gambiae* s.s. and *An. coluzzii* is more prevalent in the equatorial area, whereas both *An. gambiae* s.s., *An. coluzzii* and *An. arabiensis* are present in

tropical areas (Etang et al., 2003). The molecular species of *Anopheles gambiae* are widely distributed across the country with *An. gambiae* s.s. being the most widespread while *An. coluzzii* is more prevalent in the equatorial region of the south (Gentile et al., 2002, Nwane et al., 2011, Wanji et al., 2003, White et al., 2010). Although the two forms have been reported to be sympatric, *An. coluzzii* is mostly found in the urban areas while *An. gambiae* has a more peri-urban and rural distribution (CDC, 2008). All three chromosomal forms – Mopti, Savanna, and Forest have been reported in Cameroon. These chromosomal forms are sympatric (occurring together) in the forest-savannah transition zone. Beyond the transition zones, only the forest form is found in the dense forested area while the savannah form is present in the Sahelian zone of the country (Wondji et al., 2005, Wondji et al., 2002, della Torre et al., 2005) while the Moptic form colonises the coastal areas (Bigoga et al., 2007a, Etang et al., 2003) (Figure 2.2).

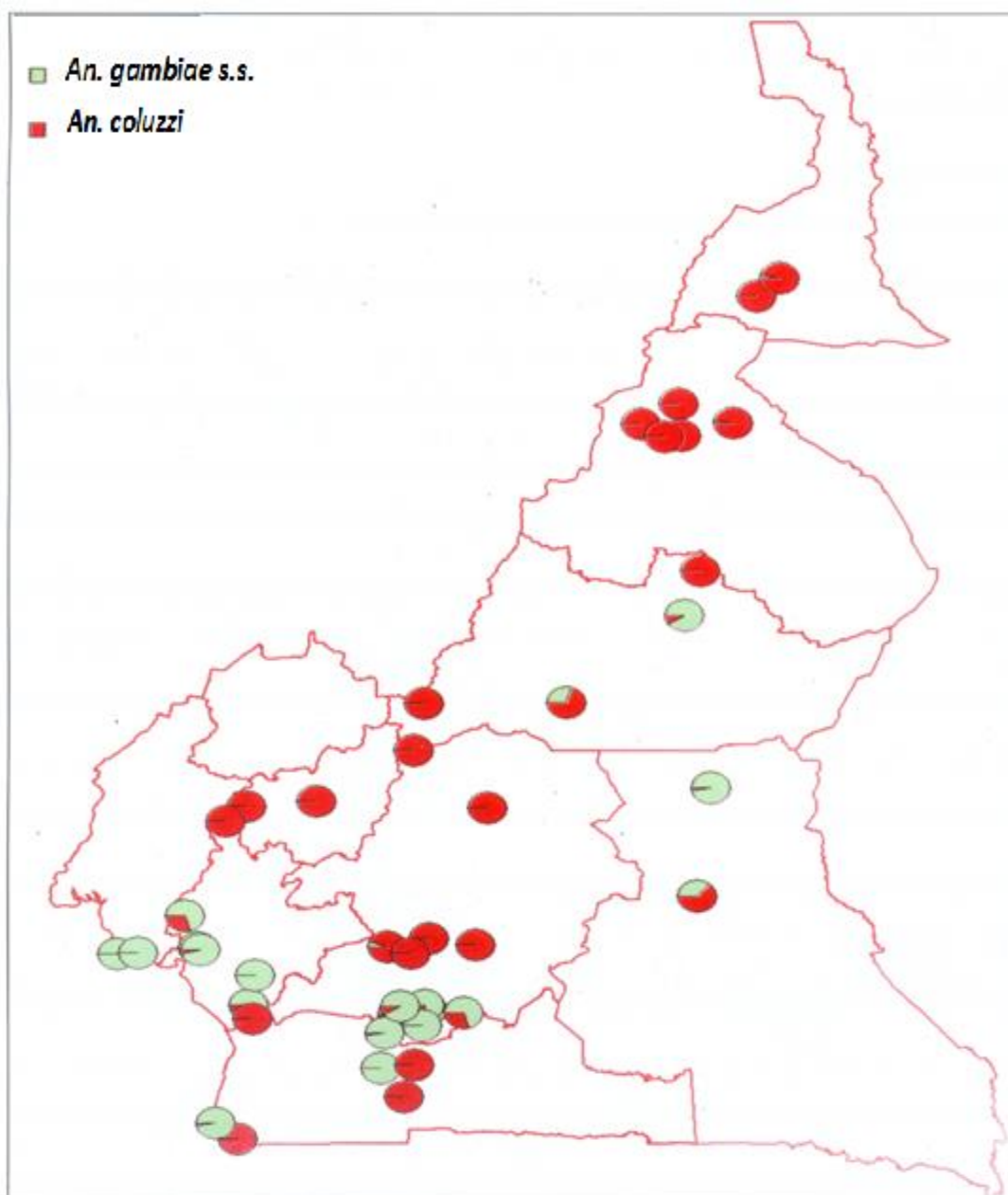


Figure 2.2: Geographical distribution and frequencies of *Anopheles coluzzii* and *An. gambiae s.s.*

National Malaria Control Programme

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Anopheles. coluzzii and *An. gambiae* s.s. are found nationwide while *An. melas* and *An. arabiensis* have a restricted distribution. *Anopheles melas* is reported only in the mangrove of Littoral and South West Regions while *An. arabiensis* is mostly found in the Sudano-Sahelian zone of the country (Antonio-nkondjio et al., 2006, Antonio-Nkondjio et al., 2005, Bigoga et al., 2007b, Etang et al., 2003, Ndong et al., 2014). *Anopheles melas*, though reported elsewhere in the country to be involved in malaria transmission, was not found to be anthropophagic in the South West Region of Cameroon by Bigoga et al. (2007); it was found to constitute only a minor proportion of the *An. gambiae* complex.

2.9.2.1 The identity of the molecular forms (*An. gambiae* s.s. and *An. coluzzii*)

Effective vector control planning and implementation can only be achieved following a thorough understanding of the identity and structure of the vector population. Before focusing on the knock down resistance profile of *An. gambiae* s.s. and *An. coluzzii* in Mbakong, it is important to take a panoramic view of efforts made towards the identification of malaria vectors so far. Morphological techniques have been used to identify members of the *An. gambiae* s.l., cytotaxonomy was used to identify *An. gambiae* s.s. as well as chromosomal forms, while different molecular techniques was used to identify M and S forms at nascent species level. Continuous refinement of the molecular tools led to the molecular forms being recognised as full species (Coetzee et al., 2013).

2.9.2.2 Adult morphology

Adult features that have been used in taxonomy include colour, dimension and pattern of the legs, wings, abdominal segments, palps, thorax, antennae etc. The presence of a dark area on the white apex of the female palps and the size of the sector spot on the wing have been used in differentiating members of the *An. gambiae* s.l. from other *Anopheles* spp. (Coluzzi, 1964). Following the work of Gillies and De Meillon, (1968) and later improved upon (Gillies and

Coetzee, 1987), a key of morphological characters was described where *An. gambiae* s.l. adult females are identified by their smooth palps with 3 pale bands on the 3rd, 4th and 5th segments; the wing field is pale with yellow or creamy markings and has pale fairly long costal spots. The number of pale spots (5) on the costal wing can be used to differentiate between *An. gambiae* and other anophelines. The femoral, tibia and the 1st tarsal segment are speckled to a variable degree. The abdomen is light brown and hairy with scales on the 8th tergite and scales on the cerci. Due to the presence of cryptic species and the ongoing speciation processes, identification using the conventional method became very difficult.

2.9.2.3 Cytotaxonomy

The need for specific identification of *An. gambiae* s.s. was stimulated by reports in Mauritius that there were two fresh-water types (A and B of *An. gambiae*) in addition to the fresh and salt water types observed in West Africa, which although sympatric are reproductively isolated (Paterson, 1964). This observation has been of paramount importance in re-orientating the morphological approach to species diagnostics, firstly in terms of the genetic criteria of speciation (Davidson, 1964), secondly to cytotaxonomy (Coluzzi, 1967); (Bruce-Chwatt, 1974) and finally to molecular taxonomy (Favia et al., 1994).

Genetic distinctness was first detected during crosses intended to reveal the genetic basis of insecticide resistance (Davidson, 1964, Davidson, 1973); F1 male sterility was sometimes observed between crosses of what were considered the same species. Eventually six species – *An. melas*, *An. merus*, *An. gambiae* s.s. (with two sibling species), *An. arabiensis*, and *An. gaudrianulatus* A – were described on the basis of reproductive compatibility; the seventh – *An. gaudrianulatus* B (Hunt et al., 1998); [now named *Anopheles amharicus* Hunt, Wilkerson & Coetzee sp. n.] (Coetzee et al., 2013) was later detected. Chromosomal inversions were shown to distinguish the species using the banding pattern of polytene chromosomes of 4th – instar

larvae and the chromosomes of the ovarian nurse cells of the semi-gravid adult females (Coluzzi, 1967).

Chromosomal inversions have been reported to enable differentiation between species through frequencies of alternative arrangements, especially involving inversions on chromosome 2. These inversions have been shown to correlate with ecological/climatic factors such as the degree of aridity of the environment, suggesting that chromosomal inversions could have specific adaptive tendencies, favouring survival under a variety of environmental conditions (Coluzzi et al., 1979, Touré et al., 1998)

Extensive studies of karyotype distributions in natural *An. gambiae* s.s. populations often revealed strong and persistent deviations from the Hardy–Weinberg equilibrium due to a deficit or even complete absence of certain heterokaryotypes, providing ample evidence against panmixia. These results led to the designation, in West Africa, of five ‘chromosomal forms’ named under a non-Linnaean nomenclature as Forest, Savanna, Mopti, Bamako and Bissau (Coluzzi et al., 1985, Touré et al., 1998, Wondji et al., 2002). The designation “form” *sensus* (Mayr, 1953) was ambiguous but it was used to denote units of uncertain taxonomic status. These forms have been characterised based on: a) the presence or absence of certain diagnostic inversions combinations (e.g. inversion 2Rb in Savanna, 2Rj in Bamako, 2Rbc/u in Mopti, 2Rd in Bissau), b) different relative frequencies of polymorphic arrangements (e.g. the standard arrangement 2R+/2L+ is almost fixed in Forest, but rare in the other forms), c) geographical distribution and ecological data (e.g. Mopti is adapted to dryer environments where it breeds all year long in irrigated fields, while Forest is exclusively found in more humid forested areas). The relative efficiencies of these chromosomal forms as malaria vectors could possibly reflect specific adaptations to eco-ethological as well as environmental parameters (Coluzzi et al., 1979, della Torre et al., 1996).

2.9.2.4 Molecular methods

The problems encountered with the aforementioned methods led to the development of techniques that ideally, offer the possibility of rapid and reliable differentiation of field-collected specimens (Favia et al., 1997, Favia et al., 2001, Lanzaro et al., 1998). The techniques included both hybridization of synthetic, species-specific DNA (Hill and Crampton, 1996) and polymerase chain reaction (PCR), using species-specific primers (Hill and Crampton, 1996). Following these studies, many markers were discovered within the mosquito genomes – microsatellite DNAs, RAPDs (Random Amplified Polymorphic DNA), and AFLP (Amplified Fragment Length Polymorphism). The other set of molecular techniques allows the detection of single nucleotide polymorphisms (SNPs) within genes (e.g. SSCP (Single-strand Conformation Polymorphism), and SNP probe in a wide variety of classical genetic markers such as cDNAs and mitochondrial DNA (Black and Lanzaro, 2001).

2.9.2.5 *M* and *S* molecular forms (presently *An. coluzzii* and *An. gambiae* Respectively.)

To help resolve the problem of the former molecular forms' identity, studies in the three decades targeted the pattern of variation observed with molecular markers that were not linked to inversions. This was largely based on the rationale that reproductive isolation would lead to genome-wide heterogeneity between chromosomal forms (Black and Lanzaro, 2001, della Torre et al., 2001, Favia et al., 1997, Favia et al., 1994, Mukabayire et al., 2001, Wondji et al., 2002). All these studies reported negligible amounts of genetic differentiation between forms with the exception of loci in the rDNA cluster, on chromosome X. rDNA loci. However, they did not support the subdivision of *An. gambiae* s.s. natural populations into five different incipient taxa, but suggested the existence of only two different entities, then referred to as molecular forms M and S (della Torre et al., 2001, Wondji et al., 2002). This method was used to identify the species of the complex and to separate M and S molecular forms in Ghana (Appawu et al.,

2004, Yawson et al., 2004), Mali (Edillo et al., 2002), Burkina Faso (Diabate et al., 2006, Diabaté et al., 2005, Edillo et al., 2002), Cameroon (Wondji et al., 2002, Wondji et al., 2005, Slotman et al., 2007).

In Mali and Burkina Faso, the exact correspondence between molecular and chromosomal forms was found, with all Mopti individuals belonging to the M molecular form (*An. coluzzii*) while sympatric Savanna and Bamako specimens showed the S pattern (*An. gambiae*) (Favia et al., 1997). This association breaks down in the more humid and forested south where only the forest form is observed but reproductive isolation of *An. coluzzii* and *An. gambiae* was maintained (Chandre et al., 1999, Coluzzi et al., 2002, della Torre et al., 2001, Wondji et al., 2002). Thus, mate recognition seems to be independent of chromosomal inversions involved in ecotypic adaptation (Favia et al., 2001). In East Africa only the *An. gambiae* (S form) has been identified so far – coinciding with the Savanna chromosomal form. This is the only chromosomal form present in East Africa, where it is extensively sympatric with *An. arabiensis* (Coetzee et al., 2000).

Gentile *et al.* (2002) reported that the geographic distribution of the molecular forms (*An. gambiae* and *An. coluzzii*) was only partly known and this was restricted to West Africa. Work in the last decade, however, revealed the presence of both molecular forms in Cameroon and Gabon (Bigoga et al., 2007b, Reimer et al., 2005, Wondji et al., 2002) and *An. gambiae* is more widely distributed than *An. coluzzii* (Gebert, 1936, White et al., 2010). In the forested areas of Central Africa, both *An. coluzzii* and *An. gambiae* share the standard chromosome 2 arrangement, which is typical of the Forest chromosomal form. Thus, the Forest form, previously described as a single panmictic unit based on chromosomal arrangements frequencies, appears to be a variant common to both *An. coluzzii* and *An. gambiae* (della Torre et al., 2001, della Torre et al., 2005, Diabaté et al., 2008, Wondji et al., 2005).

2.9.2.6 Genetic differentiation between *An. gambiae* and *An. coluzzii*

In the past few decades efforts intensified toward understanding the process of ecological diversification and lineage splitting within the molecular species especially. This efforts have increasingly been supported by the fact that each species is adapted to a particular ecotype (della Torre et al., 2001, della Torre et al., 2005, Lehmann and Diabate, 2008, Costantini et al., 2009, Manoukis et al., 2008, Simard et al., 2009). In this process, two nascent species, the M and S molecular forms were recognized (White et al., 2010). Wondji et al. (2002) had earlier reported that geographical differentiation between forest molecular forms was low despite geographical distances however, genetic differentiation between forms was very high in sympatric *An. gambiae* and *An. coluzzii* collected within the same village in Cameroon. Slotman et al. further expanded on this by studying the genetic differentiation between samples from Cameroon and Mali. This later study revealed that genetic differentiation between the Savannah *An. gambiae* and the Forest *An. gambiae* from Cameroon and Mali is very low but very high between the forest *An. gambiae* and the *An. Coluzzii* from both countries. Such findings led to the suggestion that the *An. coluzzii* may be a single entity across large distances (Slotman et al., 2007). Furthermore, though sympatric across most of their range the *An. gambiae* and *An. coluzzii* are morphologically similar and can only be separated using molecular tools. In addition, males of each molecular species create separate mating aggregations (della Torre et al., 2005, Diabaté et al., 2009).

The results clearly demonstrated the existence of a barrier to gene flow between homokaryotypic (Forest) populations of the *An. gambiae* and *An. coluzzii* in an area of sympatry. Yawson et al. (2007) while studying the *An. gambiae* and *An. coluzzii* in Ghana, supported the fact that geographic distances did not seem to play a role in differentiation between the *An. gambiae* and *An. coluzzii*, and argued that differences in ecological zones could act as barriers to gene flow.

Recent studies have demonstrated that the more ancestral *An. gambiae* (Gebert, 1936, White et al., 2010) is also a more efficient malaria vector than the *An. coluzzii* (Okoye, 2005) and shows a high frequency of the *kdr* gene frequency than the *An. coluzzii* (Antonio-Nkondjio et al., 2011, Etang et al., 2006, Nwane et al., 2011, Yawson et al., 2004). Though it had earlier been reported that the occurrence of *kdr* alleles in the *An. coluzzii* is through introgression from the *An. gambiae* (Weill et al., 2000), Reimer et al. (2005) reported that the *kdr* allele frequencies are present in the *An. coluzzii* in the island of Bioko in Gabon and absent in both *An. coluzzii* and *An. gambiae* Tiko in coastal Cameroon. Turner et al. (2005) and Turner and Hahn (2007) demonstrated that small genomic islands of significant divergence, in low recombination centromere-promiximal regions on chromosome 2L and X and a third non-centromeric region on chromosome 2R could be used to distinguish between the *An. gambiae* and *An. coluzzii* in Cameroon (White et al., 2010). Through population re-sequencing and explainable by variable signatures of selection, two differences in non-centromeric region (an island of divergence on 2R only in Cameroon and an island on 3L only in Mali) were validated and used for the identification of *An. gambiae* s.s. and *An. coluzzii* in the field (White et al., 2010).

2.10 Molecular markers

Several molecular markers have been designed to target specific DNA sequences of the mosquito genome using species-specific primers. These markers have enabled the differentiation of members within species complexes that could not be separated using previous methods.

2.10.1 Internal transcribe spacer 2 (ITS2)

Among the molecular markers used for mosquito taxonomy, the internal transcribe spacer 2 (ITS2) of the rDNA genes is useful for distinguishing between specimens belonging to closely-related species. For example, the *Anopheles maculipennis* complex (Porter and Collins, 1991),

An. quadrimaculatus complex (Cornel et al., 1996) and *An. culicifacies* complex (Goswami et al., 2005) have been analysed using this marker. Paskewitz *et al.* noted that in other complexes, composed of recently-diverged members, such as the *An. gambiae*, the ITS2 interspecies differences are minor, from 0.4 to 1.6%, preventing the use of this marker alone for taxonomic purposes (Paskewitz et al., 1994).

The molecular techniques include both hybridization of synthetic, species-specific DNA (Hill and Crampton, 1996) and PCR-based techniques using species-specific primers (Collins and Paskewitz, 1996). Using a set of primers specific for a segment derived from the rDNA cluster, the latter methodology led to a reliable and very fast identification of the members of the *An. gambiae* complex, based on the sequence divergence of the ribosomal genes in the different species. In comparison, *An. gambiae* is reported to be more variable at the level of the ITS2 than the *An. coluzzii* (Gentile et al., 2002).

2.10.2 Intergenic spacer (IGS)

The identification of diagnostic restriction site polymorphisms in the intergenic spacer (IGS) region of the rDNA segment (Scott et al., 1993), that could be used to differentiate the Mopti form from Savanna and Bamako forms in Mali and Burkina Faso facilitated the development of a fast and reliable method to distinguish these incipient species (Favia et al., 1997). This technique further refined the diagnostic test based on the IGS region by developing a direct PCR-based assay, which would provide a faster and cheaper alternative to the PCR restriction fragment length polymorphism (RFLP) test mentioned above (Favia et al., 2001). At the level of the IGS, the *An. coluzzii* is more variable than the *An. gambiae* (Gentile et al., 2002).

2.10.3 DNA barcoding using the cytochrome c oxidase I (COI) marker

Recently, DNA barcoding has been proposed as a universal method for both identifying species and uncovering biological diversity (Barrett and Hebert, 2005, Hebert et al., 2003, Hebert et al., 2004a, Hebert et al., 2004b). DNA barcoding involves the collection and databasing of DNA sequence data from a single gene region, generally a 648-bp piece of mitochondrial COI from every species. The mitochondrial genome of animals represents a better target for analysis than the nuclear genome because of its lack of introns, its limited exposure to recombination and its haploid mode of inheritance. In addition, there are robust primers that enable the recovery of specific segments of the mitochondrial genome from a broad range of animals. Sequence analyses revealed that the mtDNA (CO1) was capable of separating closely related species of *An. gambiae* complex and subpopulations of *An. gambiae* and *An. coluzzii* according to ecological zone and regions. It was shown that the molecular species do not overlap though some gene flow could still be ongoing between them, suggesting a recent expansion with the *An. coluzzii*, being more recent than the *An. gambiae* and possibly many unidentified species (Ndong, 2008). The results also indicate subdivisions within the molecular forms pointing to intraspecific divergence (Besansky et al., 1997).

2.10.4 Genetic islands of speciation

In 2005, Turner and colleagues performed a scan to map divergence between sympatric and chromosomally homogenous *An. gambiae* s.s. and *An. coluzzii* genomes from Cameroon (Turner et al., 2005). This led to the discovery of two small genomic islands of divergence (single nucleotide polymorphism) in low recombination centromere-proximal regions on chromosome 2L and X that coincided with the rDNA locus by which they had been previously defined (Della Torre et al., 2005). This finding was later confirmed in distant Mali where White *et al.* (2010) discovered a centromere-proximal region with significant divergence on chromosome

3L. These have unanimously been termed “islands of speciation” (White et al., 2010). Based on the promise offered by these new tools (SNP on chromosome 2L and 3L of the M and S forms), White et al. (2010) developed and proposed a rapid and inexpensive PCR-RFLP assay suitable for use in the field to facilitate the identification of the *An. gambiae* and *An. coluzzii* (Favia et al., 1997). All these findings culminated in raising the molecular forms to full species in 2013 (Coetzee et al., 2013). No work has so far been conducted with the aim of identifying the molecular species of these malaria vectors in the Mbakong Health area.

2.11 Malaria vector control activities in Cameroon

Pyrethroid-impregnated bed nets are a central component of the World Health Organization Global Strategy for Malaria Control (NIS, 2012). The use of insecticides hold particular promise in Africa where 80–90% of the world’s malaria cases occur, but the upsurge of resistance in the mosquito vectors to presently used pyrethroids, considered to be more effective with few side effect on man, has the potential to undermine contributions already made by control efforts (Ranson et al., 2011). Malaria prevention is heavily dependent on the use of insecticides for the control of its mosquito vector populations. Across Africa insecticide resistance in malaria vectors has become a major concern for public health authorities and national malaria control programmes (Chouaïbou et al., 2008, WHO, 2006a). The Cameroon National Malaria Control Programme (NMCP) has in the past two decade intensified efforts to prevent malaria through promoting the use of insecticide treated nets to prevent man-vector contact. Initially, this programme targeted only the pregnant women and the under five children but in 2012, more than eight million long lasting insecticidal nets (LLIN) were distributed nationwide to the general population. The purpose was to scale up the prevention efforts (NIS, 2012).

Resistance to insecticides in the malaria vector can be the result of an alternative point mutation at position 1014 in the voltage-gated sodium channel resulting in the leucine-phenylalanine

(L1014F) or a leucine-serine (L1014S) substitution termed the *kdr* alleles or it could be due to metabolic resistance, which results from the over-production of the detoxifying enzymes e.g. glutathione S-transferase, mono-oxygenase or esterase (Beaty and Marquardt, 1996, Santolamazza et al., 2008). Extensive work has been conducted on insecticide resistance in Cameroon especially within the last decade (Bigoga et al., 2007a, Chouaïbou et al., 2008, Etang et al., 2003, Reimer et al., 2008b, Wondji et al., 2005, Wondji et al., 2011, UNICEF, 2005, Reimer et al., 2008a). In 2003, it was demonstrated that *An. gambiae* complex, *An. arabiensis*, *An. gambiae* s.s. and *An. coluzzii* have developed resistance to DDT, dieldrin, permethrin, and deltamethrin to variable degree (Etang et al., 2003). Since the distribution of *kdr* alleles in West Africa had previously provided evidence of strong barrier to gene flow between *An. gambiae* and *An. coluzzii* (Chandre et al., 1999), Etang and team, tested specimens for the 'leu-phe' *kdr* mutation. Although DDT and dieldrin had once been used in this area in the 1950s for seven years (Cavaiié and Mouchet, 1962), no *kdr* mutation (leu-phe) was detected, leading to the suggestion that other mechanisms of insecticide resistance such as the 'Leu-Ser' mutation or detoxification enzymes may be at work to account for the observed resistance in the *An. gambiae* and *An. coluzzii* observed.

Two years later, the *kdr* alleles were detected in Campo in a heterozygous state (Etang et al., 2006) while Chouaïbou et al., 2008 also reported low levels of *kdr* allele frequencies in the northern cotton field. In a similar study Reimer *et al.* (2005), demonstrated the absence of *kdr* in the *An. gambiae* s.s. and *An. coluzzii* in Tiko, though it was found in higher frequencies in *An. coluzzii* in Bioko, Equatorial Guinea. In a recent multi country study, Santolamazza *et al.*, (2008) reported that both *kdr* point mutations L1014F and L1014S are present in *An. gambiae* and widely distributed, while only the L1014S mutation was reported in *An. Coluzzii*. The L104S mutation in *An. coluzzii* is restricted to the coast of the Gulf of Guinea – Cameroon, Nigeria and Benin. These mutations have recently been reported to be wide spread in the central Africa

region and have been identified in Gabon, Equatorial Guinea, Angola, Chad and Cameroon together with the L1014F mutation (Etang et al., 2007, Etang et al., 2009a, Pinto et al., 2006, Tonking and Gebert, 1947, Curtis and Lines, 1985).

2.12 Distribution of L1014F and L1014S mutations in Cameroon

In Cameroon both leucine–phenylalanine and leucine-serine mutations have been reported to occur in the same mosquito (Reimer et al., 2008a). Etang et al. (2006) observed that the L1014F is found in both *An. gambiae* s.s. and *An. coluzzii* but predominantly occurs in the *An. gambiae* s.s., whereas L1014S was only detected in the *An. gambiae*. Their co-occurrence and rise in frequency in mosquito vector populations in Cameroon is evidence of the ongoing geographical spread of both alleles invading wild *Anopheles gambiae* populations throughout Africa (Nwane et al., 2011). In a recent map of *kdr* allele distribution in Cameroon, Nwane et al. (2011) demonstrated that *kdr* allele mutations are present across all the ecological zones of the country in varying degrees. Accordingly, the L1014F frequencies are present in both the *An. gambiae* and *An. coluzzii* across all geographical areas in Cameroon except in the northern savannah where only the *An. gambiae* s.s. occurs. On the other hand, the L1014S mutation was only detected in *An. coluzzii* at Nkolbikon in the forest zone but this mutation was detected in the *An. gambiae* s.s. across all the geographical zones (Nwane et al., 2011). In a similar study, Remier et al. (2008) reported the presence of both mutations with higher frequencies in the *An. gambiae* s.s. than *An. coluzzii* populations from Bertoua in the East Region and Santchou in the West Region of Cameroon. It was further observed that the degree of resistance conferred by L1014F and L1014S varies with the insecticide in use. Both alleles were found to be associated with resistance to DDT more than to permethrin, lambda-cyhalothrin and deltamethrin (Reimer et al., 2008a). The association of *kdr* allele mutations and DDT poses a threat to the indoor residual programme currently recommended for malaria control in Cameroon. This means that

the recommendation would be turning towards using type II pyrethroids rather than type I pyrethroids or DDT where high frequencies of *kdr* are detected in *An. gambiae* populations.

A recent publication mapping the entomological resistance profile of various malaria vectors in Cameroon by the Ministry of Public Health, revealed that there is resistance development to insecticides such as DDT, Deltamethrin, permethrin, Lambda-cyhalothrin, and bendiocarb (Minsanté, 2010). These resistance species are sparsely distributed across different ecotypes and render control strategies an uphill task to planners. Apart from the insecticide pressure from the agro-industrial concerns, domestic use of insecticides especially the impregnated nets, has been ongoing for about fourteen years and could also be contributing to the development of the *kdr* mutation in the country (Nwane et al., 2011). According to the maps presented, there seems to be resistance to all the various insecticides listed in the North West Region of the country where not many studies have been carried out, including the Mbakong Health Area in the Bafut Health District.

2.13 Diagnostic polymerase chain reaction for *kdr*

Martinez-Torres et al. (1998) developed a diagnostic PCR to distinguish between resistant and susceptible *kdr* alleles found in West African strains of *An. gambiae*. This allows detection of heterozygotes for the leucine–phenylalanine mutation in field populations. The assay uses primers AgD1 and AgD2 that flank the region containing the *kdr* mutation and amplify a 293 bp product from genomic DNA. Primers AgD3 and AgD4, internal to this region, are allele specific. Primer AgD3 binds only to the resistant *kdr* allele and, when paired with AgD1, will amplify a 195 bp fragment if this allele is present in the individual. AgD4 binds only to the susceptible allele and will pair with AgD2 to produce a 137 bp band if the susceptible allele is present (Martinez-Torres et al., 1998). Ranson et al. (2000) adapted this PCR to detect the novel mutation found in the DDT/pyrethroid resistance by

substituting primer AgD3 for AgD5. This adaptation allowed the detection of heterozygotes for the leu-ser mutation of the *kdr* allele. This led to the uncovering of a resistance puzzle that could not be resolved by the former approach. No study, till date, has examined the status of *An. gambiae* and *An. coluzzii* in Mbakong.

This thesis attempts an investigation into the trends in malaria prevalence, case management as well as the status of the *kdr* mutation in the *An. gambiae* and *An. coluzzii* population in Mbakong.

2.14 Reference

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CHAPTER THREE

Trends in malaria admissions at the Mbakong Health Centre of the North West Region of Cameroon: a retrospective study

(ARTICLE 1)

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Trends in malaria admissions at the Mbakong Health Centre of the North West Region of Cameroon: a retrospective study

Ignatius C Ndong^{1,2,3*}, Mari van Reenen⁵, Daniel A Boakye⁴, Wilfred F Mbacham^{2,3} and Anne F Grobler^{1*}

1. Abstract

Background: Malaria is the leading cause of death worldwide. It is urgent to assess the impact of interventions and scaled-up control efforts. Despite reported reduction in malaria prevalence in Africa, the trends in Cameroon are not yet fully understood. The aim of this study was to investigate the trends in malaria admissions among febrile patients seeking treatment over a seven-year period (2006–2012) in an endemic area in Cameroon, hypothesizing a declining trend. This period followed changes in malaria treatment policy. The objectives were to identify possible trends in malaria admissions and to evaluate the impact of changes to treatment guidelines on the prevalence.

Methods: Data was collected through consultation and perusal of laboratory and prescription registers of the Mbakong Health Centre. Data analysis was conducted using SPSS and SAS Statistics.

Results: Analysis revealed that 4,230 febrile patients were received from 2006–2012. Of these febrile cases, 29.30% were confirmed positive. Between 2006 and 2012 confirmed malaria positive cases of those tested fluctuated, dropping from 53.21% in 2006 to 17.20% in 2008; then rising to 35.00% in 2011 and, finally, dropping to 18.2% of those tested in 2012. The prevalence in females and males across all age groups were similar: a slightly higher risk of males to have malaria (OR = 1.08, 95% CI 0.94–1.25) were not practically significant. Of those tested, the 5 to < 15 years and the 1 to < 5 years age groups were the hardest hit by malaria in the area. A practically visible and significant association was observed between the age and gender with regards to the number of malaria positive results (Pearson $\chi^2 = 153.675$, $p < 0.00001$, Cramer's $V = 0.352$). Malaria prevalence exhibited a fluctuating yet declining trend, as observed over the 28 quarters between January, 2006 and December, 2012.

Conclusions: The changes to the treatment guidelines appear to result in a declining trend as was observed between 2006 and 2008. However, malaria admissions fluctuated between 2008 and 2012. There is, therefore, a need to step up control efforts of especially the vulnerable groups, such as the very young.

Keywords: Malaria, Prevalence, Mbakong, Cameroon, Trends, Treatment, Policy

2. Background

Malaria is a complex disease that varies widely in epidemiology and clinical manifestations and remains the leading cause of death in the world [1,2]. The 207 million malaria cases reported globally in 2012 resulted in an estimated 627,000 deaths with 80% of the cases in Africa [3]. In 2010, malaria was responsible for 24% of

child deaths in Africa [4]. The world malaria report of 2013 further revealed that between 2000 and 2012 the malaria mortality among Africa children dropped by 54%. Malaria is endemic in Cameroon with a prevalence rate of 29% [5]. It is the major cause of morbidity and mortality among the most vulnerable groups: children under five (18%), pregnant women (5%), people living with HIV/AIDS (5.5%), and the poor (40%), the last of which constitutes two thirds of the total population estimated at 19 million [6]. Close to one million clinical cases of malaria occur annually, accounting for more than 50% morbidity among children under age five, 40 to 45% of

* Correspondence: Ndongicheng@yahoo.com; anne.grobler@nwu.ac.za

¹DST/NWU Preclinical Drug Development Platform, North-West University, Potchefstroom Campus, Vanderbijlpark, South Africa

²Department of Health Economic Policy and Management, School of Health and Medical Sciences, Catholic University of Cameroon, Bamenda, Cameroon
Full list of author information is available at the end of the article

medical consultations, and 30 to 47% of hospitalizations [7]. In addition, it is responsible for 49% of prenatal consultations and 59% of hospitalizations during pregnancy leading to abortions, premature labour and deliveries and low birth weight (LBW), thereby resulting in infant and maternal mortality [8,9].

The epidemiologic profile of malaria in Cameroon appears to be aligned along three geographical and climatic regions: i) an endemic and perennial transmission zone covering the southern equatorial forest, coastal and western plateau with 7–12 months of rainfall, ii) an endemic and seasonal transmission zone in the Adamawa plateau and savannah forest with 4–6 months of rainfall and iii) an epidemic and strongly seasonal zone covering the Sudano-sahelian region with seasonal transmission of 1–3 months [9,10].

The main methods for malaria diagnosis have been by clinical diagnosis and microscopy until recently, when rapid diagnostic test (RDTs) were introduced and the Cameroon government rolled out RDTs in 2012 to improve health facility diagnoses and home based management of malaria. Microscopy as a testing service is widely available (at 91% of public and 100% of private facilities), but reportedly underused, with less than half of febrile patients tested for malaria during their consultation in some countries in sub-Saharan Africa [5,11].

Over the last decade, the Cameroon government has taken a series of steps to reduce the burden of malaria. Owing to the development of resistance to various anti-malarial drugs, the government adopted the WHO recommendations of 2001 and changed the treatment policy from chloroquine to artesunate-amodiaquine (ASAQ), as the first-line treatment for uncomplicated malaria in 2004 and endorsed artemether-lumefantrine (AL) as an alternative artemisinin combination therapy (ACT) in 2006, following alleged side-effects linked to ASAQ. Under this new policy quinine and artemether injections are reserved for cases of severe malaria, and sulphadoxine-pyrimethamine for intermittent preventive treatment during pregnancy [5,12–14].

Although the impact of interventions on malaria prevalence may differ from country to country [15], there is mounting evidence across Africa that the increasing level of malaria interventions have led to a reduction in malaria prevalence from 37% in the years 1985–1999 to 17% in 2000–2007 and a consequent decline in morbidity and mortality [16,17]. This has been brought about through combining ACT and vector control using long-lasting insecticidal nets (LLIN) [4]. In Rwanda, in-patient and outpatient malaria cases in the under five age group decreased by 55% and 67% respectively and in Ethiopia by 73% and 62% following a nationwide distribution of LLIN and artemisinin-based combination therapy (ACT). In Cameroon, there has been an overall reduction from

41% in 2008, through 36% in 2010 to 31% in 2011 while malaria related deaths reduced from 29% in 2009 to 19% in 2011. In this country, free malaria treatment was started for all children under five years of age in 2011. In addition, community based management of malaria using community relay agents was started and in 2012 more than eight million free mosquitoes nets were distributed to families nationwide [18–21]. The latter was a scale up of the already existing programme of free mosquito net distribution to pregnant women and under five children who visit antenatal clinics [22–24]. Maternal mortality due to malaria also decreased from 5% in 2010 to 1% in 2011 [21]. This could lead to a change in the transmission profile of different zones [25]. Thus, the WHO in 2010 recommended parasitological confirmation of all malaria cases before treatment for patients five years and above where testing services are available [26,27]. This was geared towards reducing the unnecessary exposure to anti-malarials.

If malaria prevalence is decreasing as a result of interventions, it is expected that the number of malaria positive cases will decline in an area where such interventions are taking place. Further investigations should also be conducted to identify the proper course of treatment for malaria negative febrile cases [28,29]. In addition, the reduction in prevalence should be evident and significant across different age groups, gender and over time. The data analysed in this study spanned a seven-year period (2006–2012). Malaria resurgence is known to follow a five- to six-year epidemic cycle [30,31], which is most probably an inherent characteristic of the disease itself [32]. It is expected that interventions would have little to no effect without knowledge of driving factors such as the disease dynamics and the climate behind the cycle [33]. To some extent, knowledge of malaria prevalence trends could be useful in planning, monitoring and evaluating the level of implementation, or of interventions within a given treatment policy over time, such as the one ongoing in Cameroon. This could detect early warning signs of drug resistance development, such as treatment failure or a sustained upsurge in the number of malaria cases, despite receiving appropriate treatment. The objectives of this study were, therefore, to identify possible trends in malaria prevalence over time and in terms of season; to assess differences in malaria prevalence across gender and age groups and to evaluate the impact of changes to treatment guidelines on malaria prevalence. Furthermore, malaria drug resistance is known to develop within an average of 10–15 years [34], and thus, seven years following the introduction of ACT could be good time to investigate the trends.

The study is designed to understand the trends in malaria admissions over the period 2006–2012 following the change from chloroquine to ACT as first-line treatment and the effect of supplementary interventions in this

area. The study site is Mbakong, a rural area located in lower Bafut along the Mezam and Menchum Rivers near the Menchum fall that has been earmarked for the generation of hydro-electricity. An in-depth understanding of the malaria prevalence in patients seeking treatment in this area will not only be of importance to the local population, but for government to improve its planning strategy for scaling up of malaria control programmes in rural areas and the affluent population that would be working on the dam or tourists visiting the Menchum fall. It will provide baseline data for the prospective studies on malaria prevalence in Cameroon.

3. Methods

Study site and population

The Mbakong Health Area consists of riverine communities with GPS coordinates 6°10'18.87" N and 10°05.40" E with a head count of about 8,000 people. Mbakong falls within the Bafut Heath District of the North West Region of Cameroon (Figure 1). It lies within the Guinea Savannah region at the border of the Bafut forest on the South and the Bu forest to the north. It experiences a maximum rainfall pattern in August with a mean annual rainfall between 0 and 390 mm and a monthly temperature range of 22.49–26.34°C. Mbakong has two distinct seasons – the dry and the rainy season. The dry season starts from November to March while the rainy season starts from March through November. Human activities in this area include farming (maize and rice), game hunting and sand mining from the rivers. Sand mining is mainly carried out by young men (above 15 years of age). The women and children are involved in maize farming while all other members of the community are involved in rice farming.

Study sample & data collection

Data was collected retrospectively through consultation and perusal of laboratory and prescription registers of the Mbakong Health Centre. Information was gathered on diagnosis, age, gender and the time (months and years) using operating procedures established for this study. The data was classified into six different age groups: <1 year (0–11 months), 1 to younger than five years (1 to <5), 5 to younger than 15 years (5 to <15), 15 to younger than 45 years (15 to <45), 45 to younger than 65 years (45 to <65) and 65 years and older (≥65). In this health facility microscopy was the only tool used for diagnosis. There was unfortunately no data on the environmental factors covering the seven years considered in this study and thus there is no attempt to assess the impact of environmental factors such as rainfall or temperature on the prevalence.

Data analysis

Data analysis was conducted using SPSS Statistics V16.0 and SAS [35,36]. All clinical diagnoses for which laboratory

confirmations were requested but the results were not obtained were treated as missing data. Given that the sample was not randomly selected and cannot be established as representative of a larger population, the analyses focused on the practical significance of findings even though statistical significance is still reported [37]. Curve estimation was used to evaluate the significance of trends in the data. Pearson's Chi Square test for relationship among variables was used to evaluate the malaria prevalence across gender and age groups. Finally, proportions and odds ratios were evaluated with regards to statistical and practical significance respectively.

4. Ethical issues

The ethical clearance for this work was obtained from the Ministry of Public Health and the Institutional Review Board of the Catholic University of Cameroon, Bamenda. In order to maintain anonymity, all patients were given codes during the data extraction process and patient names were not transcribed from the register.

5. Results

The impact of changes to treatment guidelines on malaria prevalence among febrile patients seeking treatment

A total of 4,230 febrile patients sought treatment at the Mbakong Integrated Health Centre and were clinically diagnosed to be suffering from malaria from 2006–2012. Microscopy was conducted for 4,158 (98.30%) patients to confirm the presence or absence of parasites. Of these, 1,239 (29.30%) were confirmed positives with 15 patients per month on average. However, this number fluctuates dramatically from month to month. Between 2006 and 2012 confirmed malaria positive cases (taken here as a percentage of those tested) fluctuated, dropping steadily from 356 (53.21%, 95% CI 0.49–0.57) in 2006 to 93 (17.20%, 95% CI 0.14–0.20) in 2008 and then gradually rose to 244 (35.00%, 95% CI 0.31–0.39) in 2011 and finally dropped to 91 (18.20%, 95% CI 0.15–0.22) in 2012 (Figure 2a).

Piecewise regression was used to identify any significant change in prevalence when observed over the 84 months spanning January 2006 to December 2012. Figure 2b indicates an inflection point at January, 2008, corresponding to the publication of new treatment guidelines. The first regression line (Figure 2b line 1) was replicated using linear model via curve estimation and had an R^2 value of 0.827, indicating a practically significant decline (gradient was also statistically significant $p < 0.001$) in prevalence (overall statistical significance $p < 0.001$) from 2006–2008. The second regression line (Figure 2b line 2) was modelled using the same methods. However, none of the models were practically significant. These findings suggest that the implementation of the new treatment

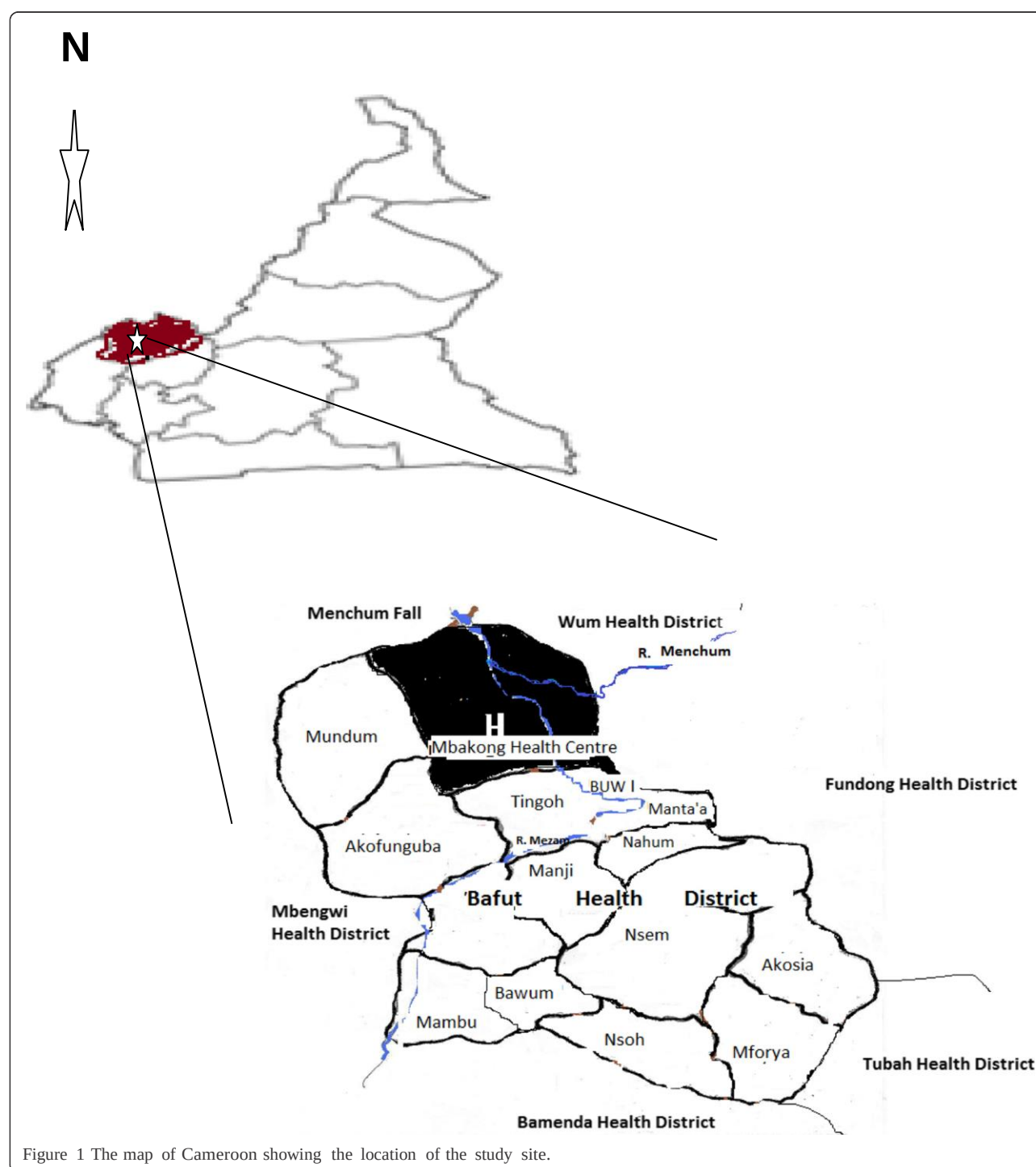


Figure 1 The map of Cameroon showing the location of the study site.

guidelines coincide with the decay of a declining trend in malaria prevalence.

Trends in malaria prevalence among febrile patients over time with reference to seasonal variation

The objective was to identify a possible seasonal trend over a 12-month period. The data used was the proportion of malaria positive cases of those tested observed

for each month over the seven-year period. As described above, curve estimation was used to evaluate trend in the data. Three models were applied to the data to evaluate trends in the data over time, namely linear, quadratic and the cubic models. While there are a multitude of models that can be used to assess trends in the data over time, those selected here were deemed the most informative. The R^2 -values (representing the percentage variance

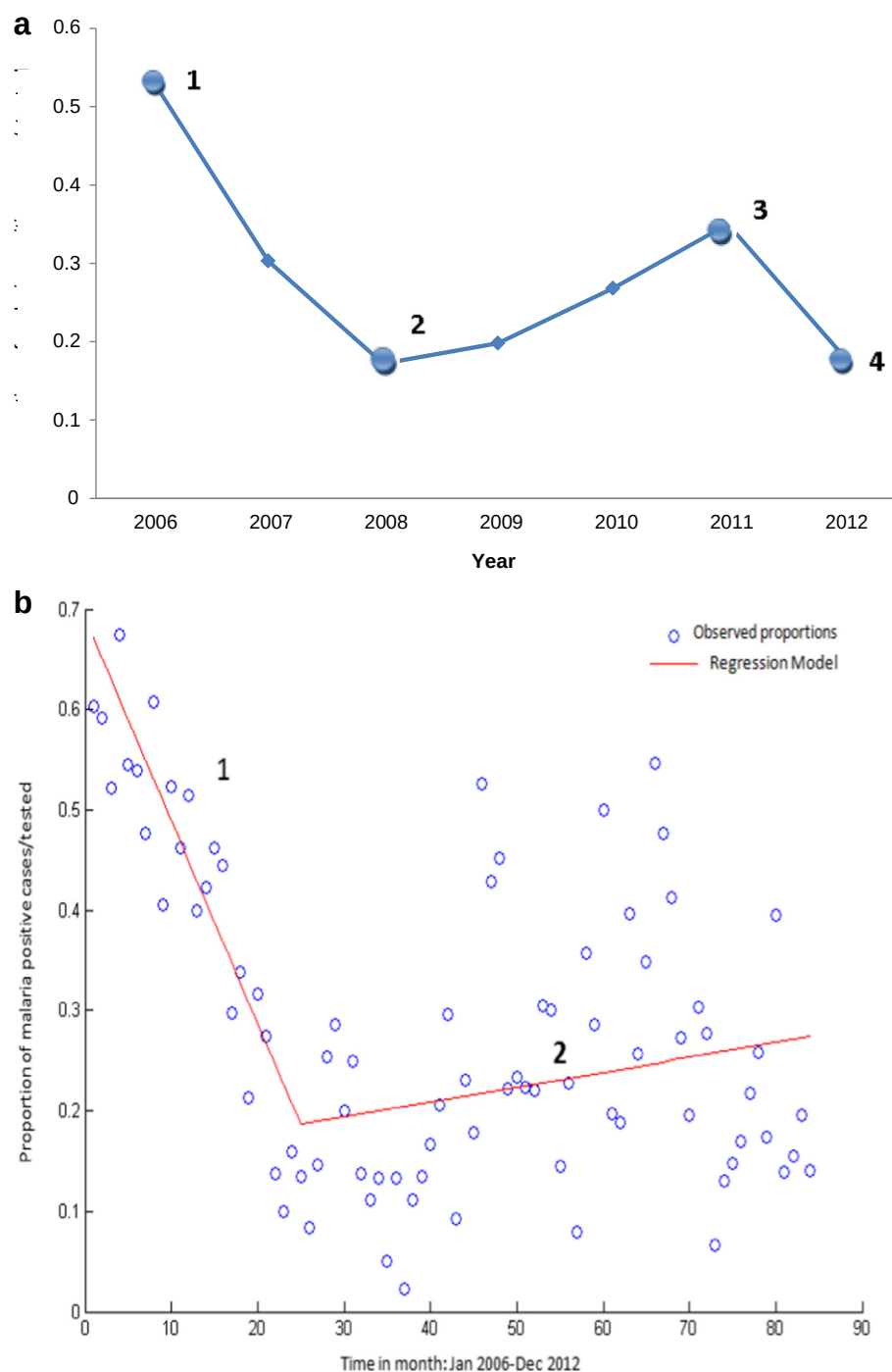


Figure 2 Distribution of malaria positive cases over seven years at the Mbakong Health Centre 2006–2012. **a)** Numbered dots indicate when different interventions were introduced: 1) implemented change in malaria treatment policy from monotherapy to ACTs, 2) New treatment guidelines made available to healthcare workers, 3) Nationwide distribution of 8 million LLIN and re-introduction of community management of malaria, 4) Roll out of RDTs for testing before treatment both at the community and health facility level. **b)** Piecewise Regression of admissions over months illustrating the change in prevalence from January 2006 to December 2012: Line 1 represents a sharp decline in malaria prevalence from 2006–2008 and Line 2 represents a gradual rise in prevalence from 2008–2012.

explained by each model) were compared to identify the more appropriate model as well as to assess the goodness-of-fit of all three models. The R^2 -values were also used to assess the practical significance of trend with values above 0.5 indicative of a practically visible trend and values above 0.8 indicative of a practically significant trend [37]. Significance testing, at the 5% confidence level, was also performed to assess the overall significance of each model as well as the model coefficients. Figure 3 illustrates the absence of a seasonal trend as none of the models were statistically or practically significant.

To conclude, no seasonal trend could be established in the data. This is contrary to popular perception that there is a major transmission season during the rainy season between May and August and minor seasons between February to April and September to November. In addition, the perception of a December-January spike, in the heart of the dry season could be of interest since the mosquito population at this time is very low and consequently low malaria transmission. However, these perceptions could not be verified in the data used for this study. There is need to investigate further malaria

prevalence in asymptomatic individuals from December through January in this area.

Curve estimation was again employed to identify predefined trends over time. The data used was the proportion of malaria positive cases to those tested observed for each month over the seven-year period; 84 observations in total. Figure 4 illustrates the three models fitted to the prevalence over time. All three models were statistically significant; however, only the cubic model explained sufficient variation to be of practical relevance ($R^2 = 0.571$, $p < 0.0001$). The linear parameter of the cubic model was both negative and significantly different from zero ($\beta = -0.042$, $p < 0.0001$). Proportions were also calculated over the 28 quarters (each year is divided up into four quarters of three months each) for the seven year period. This was done to reduce some of the variation in the observed monthly data in an attempt to identify trends. A cubic trend was found to be statistically significant and practically visible ($R^2 = 0.638$, $p < 0.0001$) (Figure 5). The coefficient for the linear component for the cubic model was significant as well as negative ($\beta = -0.132$, $p < 0.001$). Both the R^2 , as well

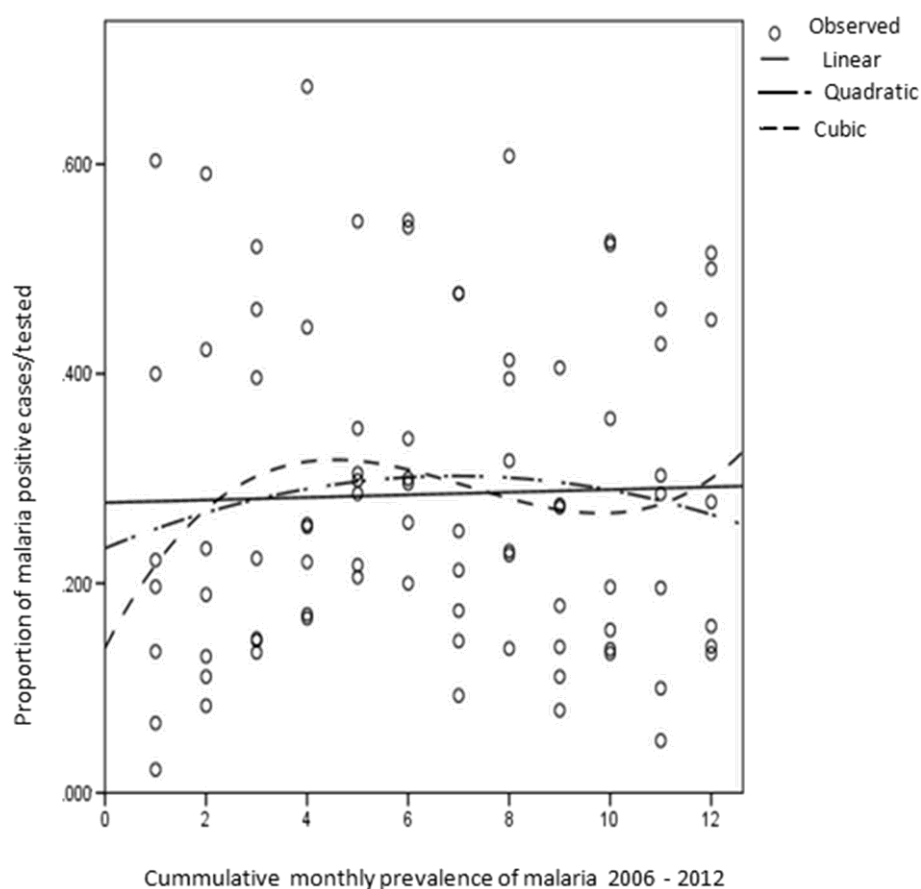


Figure 3 Three models were used to investigate seasonal trend in malaria occurrence. The graph shows monthly proportions fitted into the curve estimation model. Both confirm the absence of a seasonal trend as none of the models were statistically or practically significant.

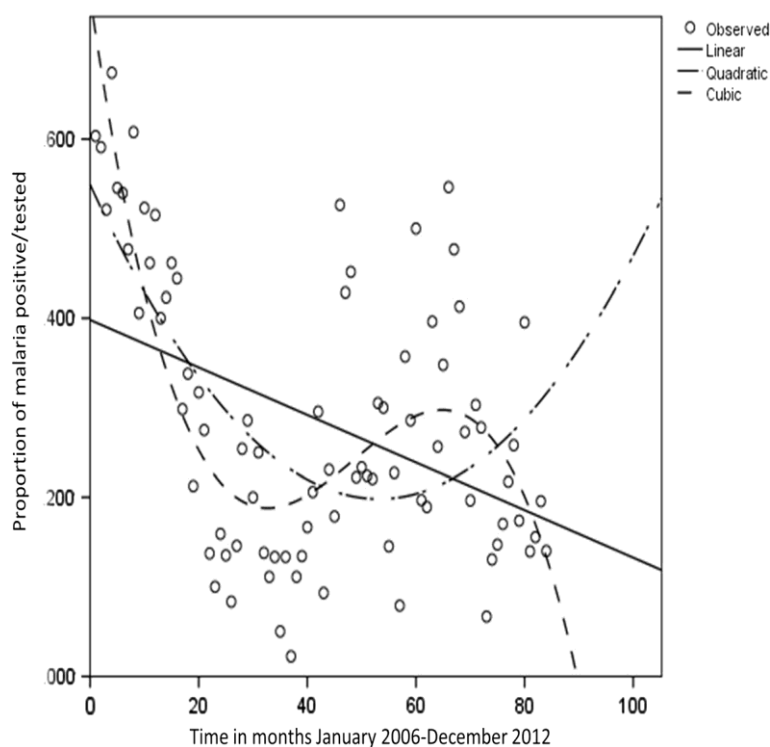


Figure 4 Graph illustrating the distribution of the proportion of positive cases according to the months of the year over time using the curve estimation model (Linear Quadratic, and Cubic). The cubic model was found to best fit the data with $R^2 = 0.638$. Time is divided into 84 monthly observations and includes Jan. 2006 to Dec. 2012.

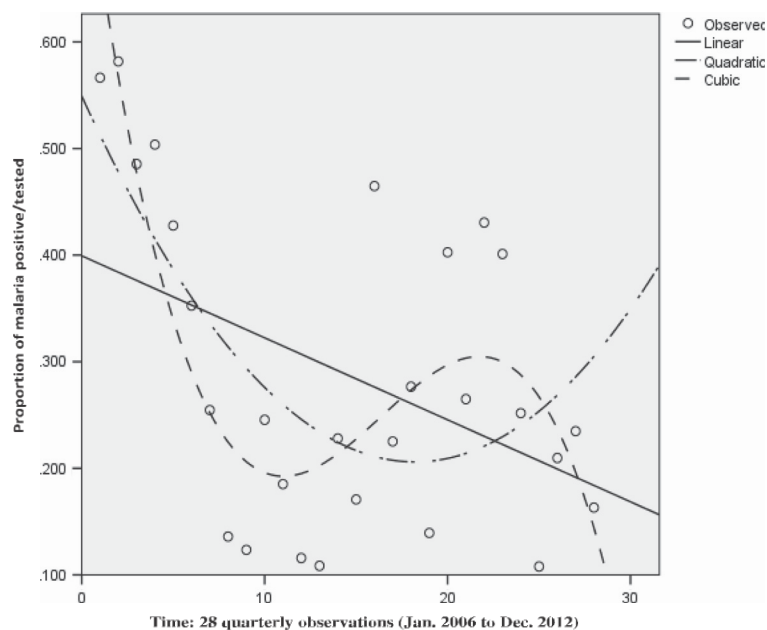


Figure 5 Graph illustrating the distribution of positive cases according to the quarters of the year over time using the curve estimation model: Linear Quadratic, and Cubic? The cubic model was found to best fit the data with $R^2 = 0.638$.

as the coefficient values were greater for the prevalence modelled per quarter, which further established a fluctuating yet declining trend.

Malaria prevalence among febrile patients across gender and age groups

The distribution of confirmed malaria cases as a percentage of those tested was evaluated across gender and age groups for the period 2006–2012. Malaria was found to have a similar prevalence in females with 798 cases of those tested (29.20%, 95% CI 0.28-0.31) and males with 441 of those tested (30.90%, 95% CI 0.29-0.33) across all age groups. The males had a slightly higher risk of contracting malaria than the females (OR = 1.08, 95% CI 0.94-1.25, $p = 0.26$) though it was not practically significant.

The number of malaria confirmed cases of those tested at the Mbakong health centre varied across different age groups. In those tested, the age groups hardest hit by malaria were the five to <15 years (296; 40.00%, 95% CI 0.36-0.44), 1 to <5 years (373; 38.26%, 95% CI 0.35-0.41) and 15 to <45 years (411; 25.23%, 95% CI 0.23-0.27). Prevalence was found to be low in the age groups younger than 1 year old children, 45 to <65 years and 65 years or older patients with 400 (19.25%, 95% CI 0.15-0.23), 60 (19.481%, 95% CI 0.29-0.33) and 106 (20.76%, 95% CI 0.13-0.28), respectively. Overall, among the 1,239 patients who tested positive for malaria, children younger than five years of age constituted 36.30% while those younger than 15 years of age constituted 60.20% in Mbakong Health Centre.

The distribution of malaria prevalence among febrile patients across age was evaluated within each gender. Evaluating whether the observed differences in age within each gender are significant required grouping of the ages into categories, similar to those used in categorizing treatment regimes. Below 15 years of age, malaria prevalence was found to be similar between the male and the female children with the respective proportions 35.4% and 35.14%, except for patients younger than one year where the malaria admissions were found to be more in males (23.76%) than females (14.64%). Above the age of 15 years malaria prevalence was higher in the females (25.23%) than the males (19.4%), except among the ≥ 65 year old patients where it was similar. The odds of having a positive result for malaria was slightly higher in the males than in the females for patients younger than one year (OR = 1.82, 95% CI 1.09-3.03 $p = 0.02$), 5 to <15 years (OR = 1.03, 95% CI 0.77-1.39, $p = 0.833$) and ≥ 65 years (OR = 1.07, 95% CI 0.41-2.78, $p = 0.98$). It is worthy of note that odds ratios were not statistically significant except for patients younger than one and none were of practical relevance [37]. In the age group 1 to <5 years and 15 to <45 years, the risk of having malaria was higher in the females

compared to the males (OR = 1.14, 95% CI 0.88-1.47, $p = 0.33$) and (OR = 1.29, 95% CI 0.95-1.75, $p = 0.097$), respectively, without any practical significance. The females in the age group 45 to <65 years had a higher risk of having malaria than the males, which was both statistically significant and practically visible (OR = 2.87, 95% CI 1.18-7.01, $p = 0.016$).

The data revealed that from 2006 to 2008 malaria prevalence among febrile patients decreased in all the age groups. A drastic drop was witnessed in the following age groups in the females: <1 year (from 54.55% in 2006 to 9.09% in 2008 and then to 0% in 2009) and 1 to <5 years (75.56% in 2006 to 15.25% in 2008), while in the males it dropped drastically in the following age groups: <1 year (50.00% in 2006 to 15.25% in 2008), 1 to <5 year old (60.87% in 2006 to 9.84% in 2008) and finally from 40 and 50% in 2006 respectively, in the age group 45 to <65 and ≥ 65 years group to 0% in 2008. Pearson's product-moment correlation coefficient was calculated to test the association between prevalence and the ages (ungrouped) of patients. The association was found to be both statistically as well as practically significant ($r = -0.55$; $p < 0.001$) with lower levels of prevalence observed in older patients.

In 2008, malaria prevalence in febrile patients seeking treatment was relatively higher in the females than males while males maintained relatively higher prevalence from 2009 through 2012. From 2008 through 2011, the age group 1 to <5 years and 5 to <14 year old children for both genders observed an increase in prevalence. In males the increase was from 9.84% in 2008 to 51.28% in 2011 in the 1 to <5 year olds while in the 5 to <15 year olds it increased from 20.56% in 2008 to 59.77% in 2011. In the females the age group 1 to <5 year old witnessed a similar rise from 15.25% in 2008 to 45.83% in 2011 while the 5 to <15 year old group witnessed a fluctuating rise from 35.85% in 2008 to 50.00% in 2011. Following the 2008 drop in malaria, the age group 15 to <45 year old has maintained a fluctuating but stable prevalence over the last five years 2008–2012. From the analysis the age groups 1 to <5 and 5 to <15 year old children seem to have been the major affected age groups during the upsurge in malaria prevalence witnessed in 2011 (Figure 6).

Pearson's Chi Square test for degree of association was used to investigate significant differences in prevalence between years, months of each year, age groups and gender (based on the number of malaria positive cases). The test revealed a practically visible association between age and gender (Pearson $\chi^2 = 153.675$, $p < 0.001$, Cramer's $V = 0.352$). A very weak association between year and age (Pearson $\chi^2 = 156.413$, $p < 0.001$, Cramer's $V = 0.159$) was observed. No significant association was found between month of the year and age (Pearson $\chi^2 = 68.469$, $p = 0.105$, Cramer's $V = 0.105$). A very weak association was found between year and gender (Pearson $\chi^2 = 13.085$, $p = 0.042$,

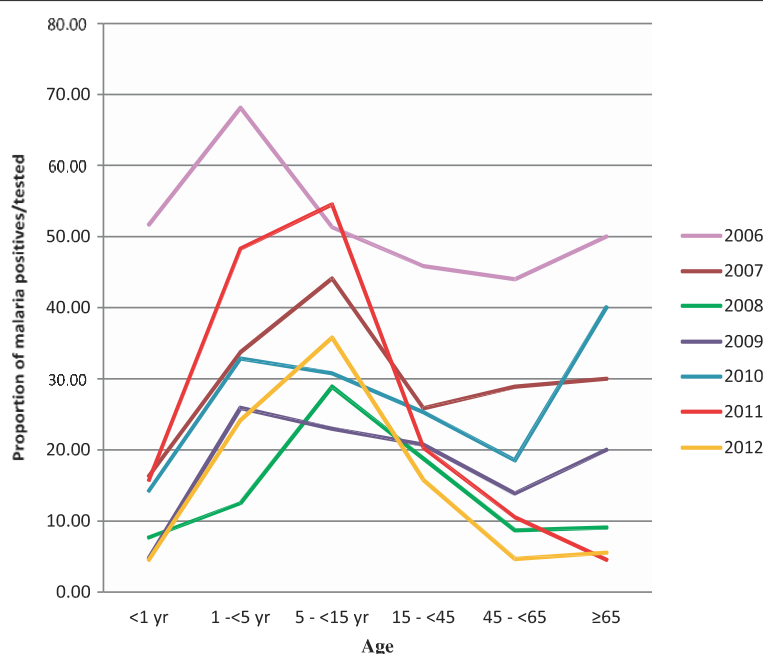


Figure 6 Graphs depicting malaria prevalence as a proportion of those tested over the study period across different age groups from 2006–2012. The age group 1 to under 5 years and 5 to under 15 years old children are revealed to be the most affected across all the years and were the hardest hit during the upsurge in 2011.

Cramer's $V = 0.105$) and no association was found between gender and month of the year (Pearson $\chi^2 = 5.510$, $p = 0.94$, Cramer's $V = 0.067$).

An analysis of the data revealed that 40/4230 (1.05%) febrile patients returned for treatment within one month of treatment for malaria at the Mbakong Health Centre with 23 (57.5%) being males while 17 were females. Of these 15 (37.5%) were <1 year infants (13 males and 2 females), 12 (30%) 15–49 years, 6 (15%) 15–14 years, 5 (12.5%) 1–4 years and 2 (5%) were 50–64 years. Twenty-six (65%) of the returning patients were children ≤ 15 years. The number of returning patients following treatment at Mbakong witness a dropped from 11 (27.5%) in 2006 to three (7.5%) in 2010 and 2011 and four (10%) in 2012. Of all the returning cases only 17 (42.5%) tested positive for malaria (nine females and eight males). Interestingly the number of returning cases increased tremendously to 28 patients in 2013 (Additional file 1).

6. Discussion

Microscopy was well utilized at the Mbakong Health Centre with 98.30% of cases tested for malaria. This demonstrates an effective use of the testing tool at the level of this health centre. This observation contrasts with earlier reports that observed that microscopy was widely available in public and private facilities, but underused, with less than half of patients tested for malaria during their consultation [5,11]. However, this conclusion is drawn from an observation in one public facility and could deviate

from this value as more facilities and different regions are included.

The results of the present study have revealed a malaria prevalence of 29.80% for tested febrile patients seeking treatment within the study period 2006–2012 in the Mbakong Health Area. The result tallied with an earlier report of a clinical trial by Mangham and colleagues [5], who observed that 29% of febrile outpatients that visit either a public or private health facility or medicine retail store were positive for malaria in Bamenda and Yaoundé. This was further supported by a report that asymptomatic pregnant women attending the Antenatal clinic in the Bafut Health District revealed a malaria prevalence of 28.4% (Engochan Mbakwa, Ignatius Ndong, Wilfred Mbacham and colleagues, 2014, personal communication). The malaria prevalence among febrile patients seeking treatment in Mbakong is lower than the 37% reported for rural areas and higher than the 15% reported for the North West region of Cameroon [20]. However, the malaria prevalence in febrile patients observed in the study was 10% lower than the 39.6% of confirmed malaria cases reported in a similar study in Ethiopia [30]. The fluctuating patterns from 2006–2012 observed in this study were very similar to those reported in studies in Ethiopia and Rwanda [30,38] with a one-year lag period. While the lowest and peak values in our study were observed in 2008 and 2011, respectively five years after the 2006 surge, those in the Ethiopian study were observed in 2007 and 2010 respectively [30]. It is not clear what the influence of

factors such as climate and environment in this regard are across the continent on the malaria cycle.

This observation closely ties in with five-year resurgence cycle of malaria. No seasonal trends were observed. Over time (taken as observations in month as well as quarters) there was a significant and practically relevant fluctuating but progressive decline in prevalence.

Since there was a significant association between the gender, age groups and malaria positives cases, future research will be required to investigate the possibility of using age group and gender to predict the future outcome of a patient tested for malaria in Mbakong and surrounding areas. However, substantial improvement seems to have been achieved with the various interventions from 2006–2012. This can be seen from the fact that annual malaria prevalence in febrile patients in 2011 was about 18% less than that of 2006 and though fluctuating, malaria admissions appear to be on the decline at the Mbakong Health centre [17].

The drop in malaria related admissions witnessed in 2012 coincides with the scaling up of the nationwide free distribution of long lasting insecticide treated nets and the roll out of RDT for community relay agents to conduct a confirmation test before treating for malaria by the National Malaria Control Programme [23,24,39]. If the latter had an effect, it seems to have been short-lived as the prevalence gradually rose in the course of 2012. This may point to the fact that the malaria prevalence in the area follows its natural cycle despite the drug and insecticide pressure [32] or that the population were excited and used the nets in the first few months and then relaxed. Alternatively, there may be a growing resistance to the insecticides used on the nets [23,24,40–42]. However, the impact of different interventions under the new policy on the level of malaria, though not yet fully investigated, cannot be under estimated. This can be seen from the fact that prevalence level within the study period even in the upsurge phase in 2011 never attained the 2006 levels. One of the setbacks in this study is the lack of data before 2006 at the health centre, making comparison to pre-2006 prevalence impossible. However, the 2006 upsurge in prevalence observed in this study coincides with those reported in a similar study in Ethiopia and Rwanda [30,38] and seems to closely match the periods between 2000–2010 during which upsurge in malaria mortality was observed in the systemic review by Murray et al. [4].

Malaria prevalence in the febrile children under five years of age is higher than those reported by Oladosu & Oyibo [43] and Alemu et al. [30], but consistent with similar findings in South Western Cameroon where higher parasitaemia in asymptomatic children has been reported [44]. Furthermore, the fact that children below age 15 years constitute 60.2% of the total malaria

positive cases detected in this health centre is in line with earlier reports that children bear the greatest burden of malaria in the world [45]. No clear pattern was found in terms of gender and age groups but correlation tests shows that as age increases, malaria prevalence tends to decrease, corroborating an earlier finding in Tanzania that malaria prevalence decreases with age [46]. This could be a result of the partially acquired immunity cumulated with age and repeated infections with the *Plasmodium* parasites [47]. While a declining trend in prevalence was witnessed by all age groups from 2006–2008, almost all the groups witnessed an upward trend from 2009–2011 except the age group 15 to <45 years, which maintained a fluctuating trend from 2009–2012. Data analyses reveal that the upsurge in malaria prevalence observed in 2011 was due to a drastic increase in prevalence in two age groups, 1 to <5 year and 5 to <15 year old children. The exact cause of this is unclear. These age groups fall within those with low partial immunity in an endemic area. The findings in this study suggest that, amongst those tested, the most affected groups are children and women. This result is in sharp contrast with the report by Alemu et al. [30] where men were the most affected. Although farming is the common denominator in the most affected groups in both studies, it is not yet clear how this contributes to the high prevalence in this young age group.

Though the odds of having positive results for malaria alternated between males and females from one age group to the other the effect size was small. Nevertheless, the chances of having malaria were found to be generally higher in the males than the females. However, females in the age group 45 to <65 years had a significantly higher risk of a positive malaria result. This was supported by a practically visible effect ($p = 0.026$ OR = 2.87234).

Though there was dropped in the number of returning cases treated for malaria at the Mbakong Health Centre from 2006–2012 which could be attributed to various interventions under the present policy. An interesting picture was observed in the high number of return cases in 2013, which was beyond this study period. Though 42.5% of the returning febrile patients tested positive for malaria, there is, however, no clue than this return rate of 1.05% was due to treatment failure using ACT since there was no patient follow up in this study. This could be a pointer that there is need to monitor for treatment failure at this health centre. This study acts as a baseline for future studies on the efficacy of ACT and other interventions.

7. Limitation of the study

The findings of this study are limited to the Mbakong health centre and based on clinical and microscopic diagnoses records of febrile patients considered retrospectively over a seven-year period 2006–2012. Prevalence

of malaria earlier than 2006 is not known as records before this period were not available. Data on asymptomatic malaria in adults is not captured in this study as it focuses on hospital records of essentially febrile patients seeking treatment.

8. Conclusions

The findings of this study reveal that the malaria admissions in Mbakong have shown a fluctuating, but declining trend over the last seven years with a practically relevant significance. The Mbakong Health Centre seems to have witnessed a significant decline in the malaria admissions in the different age groups. Though it is not clear whether the observed decline was due to the intervention under the current policy or part of the natural cycle of malaria, the impact of the new treatment policy cannot be under estimated. There is need for control efforts to be stepped up and for establishing a monitoring scheme with specific reference to the vulnerable groups.

One of the cyclic phases of the malaria disease with a declining trend seems to have been witnessed within this seven year period of 2006–2012. However, the study period needs to be extended and the number of facilities increased for meaningful conclusions to be drawn. Nevertheless, this study provides a baseline for malaria prevalence studies to be conducted in this area in future.

9. Additional file

Additional file 1: Table depicting the number of febrile patients who returned to the Mbakong health centre within one month of an initial malaria treatment.

10. Competing interests

The authors declare that they have no competing interests.

11. Authors' contribution

ICN: Conceived the study and experimental design, data collection, analysis and interpretation, preparation of the manuscript. WFM: Contributed substantially to conception, study design, interpretation of data, and critically reading the manuscript. AFG: Contributed to designing the molecular analysis and interpretation of the data as well as critically revising the manuscript. DAB: Experimental design of the study, analysis and critically revised manuscript for intellectual content. RvM: did the statistical analysis as well as critically reviewed the manuscript for intellectual content. All authors read and approved the manuscript.

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13. Author details

¹DST/NWU Preclinical Drug Development Platform, North-West University, Potchefstroom Campus, Vanderbijlpark, South Africa. ²Department of Health Economic Policy and Management, School of Health and Medical Sciences, Catholic University of Cameroon, Bamenda, Cameroon. ³Laboratory of Public Health Research Biotechnologies, Nkolbisson, University of Yaoundé I, Yaoundé, Cameroon. ⁴Noguchi Memorial Institute of Medical Research, University of Ghana Legon Accra, Accra, Ghana. ⁵Statistical Consultation Services, North-West University, Potchefstroom Campus, Vanderbijlpark, South Africa.

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CHAPTER FOUR

TRENDS IN MALARIA CASE MANAGEMENT FOLLOWING CHANGES IN THE TREATMENT POLICY TO ARTEMISININ COMBINATION THERAPY AT THE MBAKONG HEALTH CENTRE, CAMEROON 2006-2012: A RETROSPECTIVE STUDY.

(ARTICLE 2)

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Trends in malaria case management following changes in the treatment policy to artemisinin combination therapy at the Mbakong Health Centre, Cameroon

2006-2012: a retrospective study.

Ignatius C Ndong^{*a, b,c}, Mari van Reenen^e, Daniel A Boakye^d, Wilfred F Mbacham^{* b,c}, Anne F Grobler^a

- a) DST/NWU, Preclinical Drug Development Platform, North-West University, Potchefstroom Campus, South Africa*
- b) Catholic University of Cameroon, Bamenda*
- c) Laboratory of Public Health Research Biotechnologies University of Yaoundé I, Cameroon,*
- d) Noguchi Memorial Institute of Medical Research, University of Ghana Legon.*
- e) Statistical Consultation Services, North-West University, Potchefstroom Campus, South Africa*

E-mail:

*Corresponding author:

Ndong Ignatius Cheng: Ndongicheng@yahoo.com, 25044672@nwu.ac.za,

Mari van Reenen: 12791733@nwu.ac.za

Daniel A Boakye: dboakye@noguchi.mimcom.org

Wilfred F Mbacham: wfmbacham@yahoo.com,

Anne F Grobler: anne.grobler@nwu.ac.za

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Author: Ignatius C Ndong Mari van Reenen Daniel A Boakye Wilfred F Mbacham Anne F Grobler

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Abstract

National malaria treatment policies are devised to guide health professionals and to facilitate diagnosis and case management. Following the recommendations of the WHO, Cameroon changed its malaria treatment policy from monotherapy to artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated malaria. We report an investigation into trends of case management following this change in policy. Data was collected retrospectively, through consultation and perusal of laboratory and prescription registers of the Mbakong Health Centre. Analysis of data was done using SPSS and SAS Statistics. Data presented herein demonstrate that from 2006-2012, a total of 2,484 (58.7%) of the total prescriptions included an anti-malarial, 1,989 (47.0%) included an antibiotic and 1,935 (45.7%) included an antipyretic. The anti-malarials prescribed were Anti-malaria combination therapy (ACT) - 1,216 (47.6%), quinine 1,044 (40.8%) or SP 296 (11.6%). Of the 1,216 patients prescribed an ACT, 441 (36.3%) had a positive malaria parasite confirmation, 746 (61.3%) were negative for plasmodium. Overall, 29 patients (2.4%) were treated either with an ACT without any test performed. Quinine intake was recorded in 566 (54.2%) patients positive for plasmodium. ACT prescription increased from 23% in 2007 to between 44%-45% in 2008-2009. During this period there was a corresponding drop in the prescription of quinine from 38% in 2007 to 13% in 2009 ($r = -0.43$, $p > 0.05$). Sulphadoxine-Pyrimethamine (SP) was restrictively prescribed to women of childbearing age (97.0%) after 2008. Antibiotics prescription dropped from 53.7% to 39.3% from 2010-2012. The odds of being prescribed an antibiotic was significantly higher in patients with a malaria negative result compared to malaria positive patients ($OR=6.12$, $CI\ 4.74-7.91$, $p < 0.00001$). Overall, there is an over-treatment of malaria, thus departing from the WHO guidelines of appropriate treatment. Although there is an overall increase in the prescription of ACT, less prescription of quinine and a noticeable restrain from prescription of SP to febrile cases, the old practice was still rampant. There is need for healthcare workers to adhere to guidelines in order to enhance

the rational use of drugs to achieve appropriate treatment of uncomplicated malaria according to WHO guidelines.

Key words: Trends, malaria, artemisinin-based combination therapy, quinine, sulfadoxine-pyrimethamine, case management, policy, Mbakong, Cameroon

1. Background

Malaria remains a major public health burden in tropical Africa and in the world. It affects mostly children under the age of five and pregnant women. Since 2001, all countries in Africa have progressively adopted and implemented the artemisinin-based combination therapy (ACT) as first-line in the management of uncomplicated malaria through the use of revised therapeutic guidelines (WHO, 2006). This change in guidelines was stimulated by the growing concerns that the malaria parasites had become resistant to chloroquine in most parts of Africa (Talisuna et al., 2004). In the 1990s the malaria epidemiologic map of Cameroon revealed that chloroquine resistance was wide spread while quinine resistance was restricted to the northern regions of the country (Brasseur et al., 1988, Brasseur et al., 1992).

In 2004, following the WHO recommendations, the Cameroon government changed its malaria treatment guidelines from monotherapy to ACT with artesunate-amodiaquine (ASAQ) as the first-line treatment for uncomplicated malaria (NMCP, 2004). ASAQ is highly subsidised by the government compared to other anti-malarials. In 2006 artemether-lumefantrine (AL) was introduced as an alternative ACT in the proportion of ASAQ (75%): AL (25%). Quinine (Q) and artemether injectables are reserved for severe malaria while sulfadoxine-pyrimethamine (SP) is reserved strictly for intermittent preventive treatment during pregnancy (IPTp). This policy was enforced through a series of training and public education workshops for health professionals. In 2008 the Ministry of Public Health republished her guidelines alongside frequently asked questions to assist in the implementation of the national guidelines (Sayang et al., 2009, Minsanté, 2008, Mangham et al., 2012).

The 2008 guidelines provided an algorithm for the health caregiver to quickly assess and distinguish between uncomplicated and severe malaria (NMCP, 2008). Though the guidelines did stress that the identification of *Plasmodium* parasites in blood films was a conclusive diagnosis for malaria in patients more than 5 years, it however further stated that the absence of parasite did not rule out malaria. That caveat opened up possibilities of presumptive therapy and poor adherence to the test results. This was widely encouraged throughout Africa prior to the advent of rapid diagnostic tests (RDTs), (Ansah et al., 2010, Mangham et al., 2012, Reyburn et al., 2007).

Studies have demonstrated that malaria is over treated in Tanzania (Reyburn et al., 2007), Angola (Rowe et al., 2009), Kenya (Juma and Zurovac, 2011) and recently in Cameroon (Mangham et al., 2012) where 70% of febrile patients prescribed ACT are negative for malaria. Interventions to evaluate the use of RDTs and improve on adherence to guidelines to reduce the over prescription of anti-malarials in Ghana (Ansah et al., 2010), Uganda (Kyabayinze et al., 2010), Kenya (Skarbinski et al., 2009), Tanzania (Ngasala et al., 2008), Zambia (Yeboah-Antwi et al., 2010), Nigeria (Mangham et al., 2011) and Cameroon (Mangham et al., 2012) show that between 35-85% of RDT negative patients are still treated with anti-malarials (Chandler et al., 2012). These efforts have been conducted in most instances to evaluate the level of implementation of the national treatment policy. It has been documented that when presumptive treatment is practiced the proportion of patients who receive appropriate treatment are reduced (Mbonye et al., 2013). In line with the 2010 WHO guidelines which recommend a parasitological confirmation for suspected malaria in children of 5 year and above, the Cameroon government in 2012 distributed RDTs to all public facilities and relay agents for home-based management of uncomplicated malaria (MINSANTE, 2009, WHO, 2010). Mangham and colleagues recently reported an improvement in the use of ACT and quinine compared to an earlier report by Sayang and colleagues, which revealed that the uptake of ACT was very low (Sayang et al., 2009, Mangham et al., 2012,). Furthermore, the

Demographic Health Surveys in 2011 had demonstrated that though unsubsidised, AL was most often prescribed compared to the subsidised ASAQ (Minsanté, 2009, Ngum et al., 2010, NIS, 2012). Increasingly, it is becoming clear that just making available the testing tools does not ultimately modify provider practice or translate into improving quality of care but providing additional support services to the health care providers could be of help (Chandler et al., 2012, D'Acremont et al., 2011, Williams et al., 2008, Achonduh et al., 2014). A recent study revealed that the implementation of RDTs as well as interventions tailored to enhance the translation of health worker knowledge into prescribing practice greatly reduced the over prescription of antibiotics and improved the quality of patient care (Mbacham et al., 2014).

A retrospective investigation into malaria case management at the Mbakong Health Centre reveals that malaria is still grossly over diagnosed and treated through the overuse of anti-malarials and antibiotics. This study examines records spanning over a seven year period (2006-2012) with microscopy as the only testing service available.

2. Method

2.1 Study Area

The Mbakong Health Area with coordinates 6°10'18.87"N and 10°05'05.40" is located in the Bafut Health District. It is a riverine community with a population of 8,000 inhabitants. The Health Area experiences a Guinea Savannah vegetation with a mean annual rainfall of 390mm and a monthly temperature range of 22.49-26.34°C. The population of this area is mainly involved in subsistence agriculture, hunting and sand mining from both the Menchum and Mezam rivers (Ndong et al., 2014).

The Mbakong Health Centre is a public facility where patients pay for all health services at a subsidised rate. However, in 2011 the government implemented a free treatment programme for all under five children suffering from uncomplicated malaria and intermittent preventive

treatment in pregnancy (IPTp). Drugs are supplied to this health centre through the North West Special Fund for Health (NWSPH) at subsidised rates with funds from the community, government as well as contributions from development agencies. The participation of the NWSFH in the drug distribution chain has over the years greatly reduced the likelihood of stock outs.

2.2 Data collection and analysis

Only paper records were available as data sources. Information was retrospectively gathered on diagnosis, treatment, age and gender from the hospital records (mainly the laboratory and prescription registers) according to established operating procedures. In this health facility, microscopy was the only tool used for diagnosis over the study period from 2006 to 2012 (Ndong et al., 2014). Thick and thin blood films were used to test for malaria parasites while bacterial infections were detected using a Gram stain as well as wet stool preparations. Each prescription was counted as a data unit. Those patients who came back within one month of treatment were treated as returning cases. To limit errors, data extraction was double checked. Data analysis was conducted using SPSS Statistics V16.0 and SAS (SAS, 2011, verwysing, 2011). Significance testing was performed at a 95% confidence level. Pearson's Chi Square test as well as odds ratios for relationship among variables were used to evaluate the effects of factors such as gender and age on the prescription received.

2.3 Ethical issues

Ethical clearance for this work was obtained from the Ministry of Public Health and the Institutional Review Board of the Catholic University of Cameroon, Bamenda. In order to maintain anonymity, all patients were given codes during the data extraction process and patient names were not transcribed from the registers.

3. Result

3.1 Malaria diagnosis:

The Mbakong Health Centre registered 4,230 febrile patients clinically diagnosed with malaria from 2006 to 2012 [2,776 (65.6%) female and 1,454 (34.4%) male]. Clinical diagnosis revealed that 3,933 (93.9%) patients suffered from uncomplicated malaria while 297 (7.0%) suffered from severe malaria. Microscopy was conducted for 4,158 (98.3%) patients. Of all the febrile patients attended to, 1,239 (29.3%) were confirmed to be malaria positive, 2,919 (69.0%) malaria negative while 72 (1.7%) were not tested. Apart from the request for malaria test, stool and urine analyses were requested in association for 3,747 (88.6%) febrile patients. Of these, 331 (7.83%) were found to be positive for bacterial infections.

3.2 Malaria case management:

Data presented herein demonstrate that from 2006 to 2012, a total of 2,484 (58.7%) of the prescriptions dispensed to patients were for anti-malarials, 1,989 (47.0%) were for antibiotics and 1,935 (45.7%) were for antipyretics. The anti-malarials prescribed were ACT 1,216 (47.6%), quinine 1,044 (40.8%) or SP 296 (11.6%). Prescription of the different anti-malarials was done using various combinations as follows (excluding the antipyretics as they accompanied most of the anti-malarials prescriptions) ACTs alone, 556 (13.1%), ACTs + antibiotics (AB) 589 (23.9%), quinine (Q) only 552 (13.1%), Q + AB 849 (20.1%), ACT + Q, 47 (1.8%) and ACT + Q + AB - 28 (1.1%). Sulfadoxine Pyrimethamine (SP) was prescribed in the following combinations, SP only 180 (4.3%), SP + Q, 552 (13.1%), SP + ACT, 12 (0.3%), SP + ACT + AB, 3 (0.1%), SP + Q + AB 4 (0.1%), SP + AB 85 (2.0%) and SP + Q + ACT 1. A total of 782 (18.5%) of the patients were prescribed only antibiotics. The chance of being prescribed a combination of anti-malarials and antibiotics was mostly higher in malaria positive patients compared to malaria negative patients.

3.2.1 Prescription of ACT: Of the 1,216 patients who were prescribed ACT, 441 (36.3%) were confirmed to be malaria positive, 746 (61.3%) were confirmed to be malaria negative while 29 patients (2.4%) were treated without a test. The use of ACT in malaria case management fluctuated in an alternating but declining pattern with the use of quinine during the study period (Figure 1). ACT prescription initially dropped from 175 (25.2%, 95% CI 0.22-.028) in 2006 to 141 (22.8%, 95% CI 0.19-0.26) in 2007 and then rose to a peak of 242 (44.8%, 95% CI 0.41-0.49) in 2008 and dropped to 96 (19.2%, 95% CI 0.16-0.23) in 2012. The odds of being prescribed an ACT was slightly higher in malaria positive patients than those with negative results (OR=1.89, 95% CI 1.57-2.28 $p<0.00001$) with no practical significance. Antibiotics and antipyretics prescription remained high throughout the study periods.

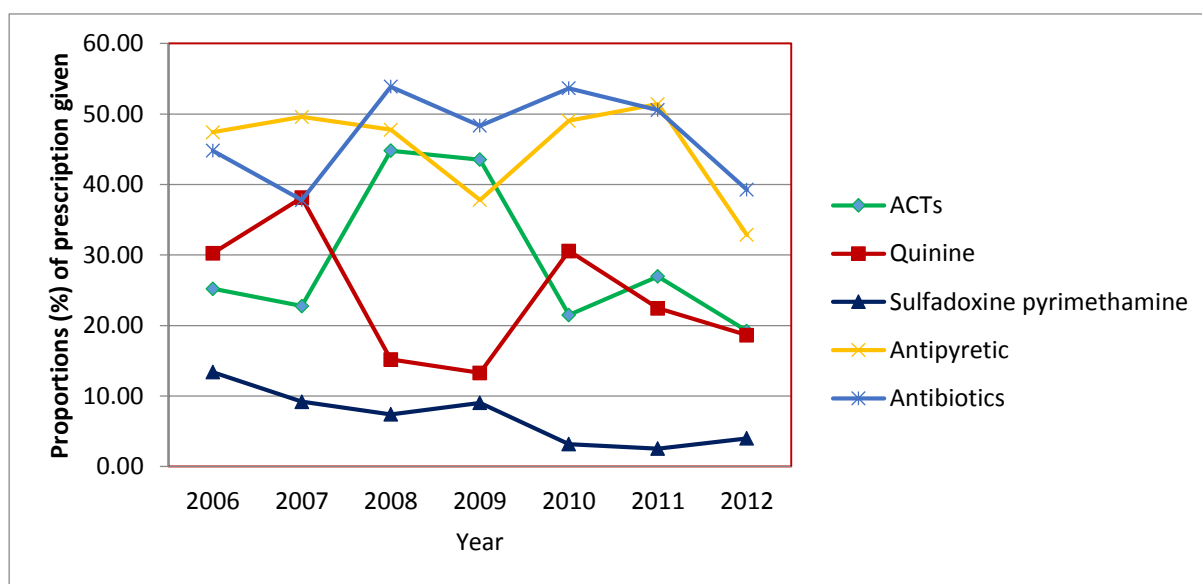


Fig 1: Graphical representation of trends in the prescription of treatment for the management of febrile illnesses. *Each data point represents the annual proportion of the patients treated with a particular drug regimen for a given year.*

The odds of being prescribed an ACT was significantly higher in the male patients than in the female patients from 2006-2012 (OR=1.80, 95% CI 1.59-2.09, $p<0.0001$). ACT prescriptions

received by both male and female patients from 2006-2012 maintained a fluctuating pattern (Table 1)

3.2.2 Prescription of quinine: Of the 1,044 patients who were prescribed quinine, 566 (54.2%) were confirmed malaria positive, 465 (44.5%) malaria negative while 13 (1.2%) were treated without a test. The odds of being prescribed quinine was higher in patients with a malaria positive result compared to those with a negative result (OR=3.37, 95% CI 2.8-4.05 $p < 0.00001$) with a practically visible association. The prescription of quinine to patients witnessed a fluctuating pattern, from 210 (30.3%, 95% CI 0.27 - 0.34) in 2006, it reached its peak in 2007 with 236 (38.1%, 95% CI 0.34-0.42) and then began to drop. The lowest quinine prescriptions were recorded in 2008 and 2009 coinciding with the highest level of prescription of ACT. This also followed the republication and the release of the 2008 treatment guidelines. Over the seven years under study, quinine prescription to female and male patients fluctuated considerably (Table 1). ACT and quinine prescriptions were observed to fluctuate in a practically visible alternating pattern. This pattern is not statistically significant and could most likely be due to the small sample (Kendall's Tau b, $r = -0.43$, $p > 0.05$, $N = 7$). All anti-malarials were prescribed in the correct doses over the appropriate time period. Quinine was usually prescribed to be taken over a seven day period. This was in line with the guidelines, however, it could not be verified within the scope of this study whether the patients did complete the prescribed treatment as there was no follow up of patients.

3.2.3 Prescription of sulfadoxine pyrimethamine (SP): Of the 296 patients who were prescribed SP, 54 (18.2%) were confirmed to be malaria positive, 238 (80.4%) were malaria negative while 4 (1.3%) were treated without a test. The odds of being prescribed an SP to patients with negative results were higher compared to patients with positive results (OR=4.20, 95% CI 2.53-6.95, $p < 0.00001$). Across gender, female patients received significantly more SP, 287 (97.0%) than male patients (9 (3.1%)) [$\chi^2 = 134.595$, $p < 0.0001$, $df =$

2]. The proportions of the total SP prescribed dropped from 87 (19.1%) in 2006 to 18 (2.5%) in 2011 in women. On the other hand SP prescription in males compared to the total SP dropped from 6 (2.0%) in 2006 to 1 (0.3%) in 2008 and from then up to 2012, no SP was prescribed to male febrile patients.

3.2.4 Prescription of antibiotics (AB): Of the 1,989 patients treated with antibiotics only 278 (14.0%) had a gram positive stain for bacterial infections while 1,711 (86.0%) had a gram negative result or were treated without a test. Annual prescriptions of antibiotics exceeded 40%, except in 2007 and 2012 with proportions of 37.8% and 39.3% respectively. From 2010 to 2012 a consistent drop in the proportions of antibiotics prescribed was observed from 537 (53.7%, 95% CI 0.50-0.58) to 196 (39.3%, 95% CI 0.35 -0.44) (Fig 1). Of all those who were prescribed antibiotics, 524 (26.3%) were confirmed malaria positive, 1,447 (72.7%) were confirmed malaria negative while 18 (0.9%) were treated without a test. The odds of being prescribed an antibiotic was higher in patients with a malaria negative result compared to patients with positive results (OR=6.12, CI 4.74-7.91, $p < 0.00001$), with a practically significant association. Within those treated, analysis revealed that females were prescribed more antibiotics 1,254 (63.1%) than males 735 (37. 0%) ($\chi^2 = 13.65$, $p = 0.001$, $df = 2$)(Table 1). Over the years females were prescribed relatively more anti-malarials than males.

Table 1: Distribution of treatment prescribed as per gender

Number of patients treated within each drug regimen (annual proportion of patients prescribed a particular drug)								
	ACT		Quinine		Antibiotics		SP	
Year	Female	Male	Females	males	females	males	females	males
	n = 678	n = 538	n = 668	n = 376	n = 1254	n = 735	n = 287	n = 9
2006	103(14.8)	72(10.4)	125(18.0)	85(12.2)	194(42.6)	117(48.9)	87(19.1)	6(2.5)
2007	80(12.9)	61(9.8)	134(21.6)	102(16.5)	145(35.9)	89(41.4)	55(13.6)	2(0.9)
2008	135(25.0)	107(19.8)	53(9.8)	29(5.6)	171(50.6)	120(59.4)	39(11.5)	1(0.5)
2009	115(21.2)	121(22.3)	61(11.2)	11(2.0)	161(44.6)	101(55.8)	49(13.6)	0
2010	77(12.3)	58(9.2)	127(20.2)	65(10.4)	222(52.0)	115(57.2)	20(4.6)	0
2011	108(15.3)	83(11.7)	97(13.7)	62(8.8)	227(50.7)	131(50.4)	18(4.0)	0
2012	60(12.0)	36(7.2)	71(14.2)	22(4.4)	134(39.1)	62(39.7)	19(5.5)	0

Prescription pressure of different regimens as per gender over the study period 2006-2012.

Values in parentheses represent the annual proportion of patients who received a particular drug regimen.

3.2.5 Prescription patterns across different age groups and gender: Across different age groups the prescription of ACTs was observed to decline with age with 254 (61.2%) in children less than one year, 402 (40.5%) in the one to less than 5 years and 13 (12.2%) in the ≥65 years. Quinine prescription was found to be high in the children age five to <15 years 249 (33.0%), one to <5 years 322 (32.5%) and in adults forty five to <65 years 81 (25.9%) and fifteen to <45 years 324 (19.7%).

SP was mostly prescribed to people in the age group fifteen to <45 years 273 (16.6%), and in one to less-than 5 year old children 5 (0.5%). Similarly, the prescription of antibiotics and antipyretics revealed a decreasing pattern as age increased. This imposed a relatively higher drug pressure (proportion of patients prescribed a particular drug) throughout all age groups. For all confirmed malaria cases, a significant difference was observed between males and females across all the prescriptions except for quinine where the difference was not significant

(Table 2A). The difference between prescriptions made to both males and females malaria negative cases was significant across all drugs used (Table 2B)

Table 2: Distribution of treatment prescribed within gender for febrile patients tested for malaria:

A) Confirmed positive malaria cases, B) Confirmed negative malaria cases

A

Number of prescriptions within the gender (% of patients receiving treatment within each gender)						
	Female	Male	Total prescribed	Pearson χ^2	df	p-value
ACTs	263(33.0)	178(40.4)	441(35.6)	7.561	2	0.023
Quinine	372(46.6)	194(44.0)	566(45.7)	1.100	2	0.577
SP	53(6.6)	1(0.2)	54(4.4)	31.653	2	<0.001
Antipyretic	442(55.4)	284(64.4)	726(58.6)	11.217	2	0.004
Antibiotics	332(41.6)	192(43.5)	524(42.3)	4.151	2	0.125

B

Number of prescriptions within gender (% of patients receiving treatment within each gender)						
	Female	Male	Total prescribed	Pearson χ^2	df	p-value
ACTs	400(20.7)	346(35.1)	746(25.6)	71.357	2	<0.0001
Quinine	290(15.0)	175(17.7)	465(15.9)	3.747	2	0.154
SP	232(12.0)	9(0.9)	241(8.3)	106.027	2	<0.0001
Antipyretic	701(36.3)	470(47.6)	1171(40.1)	35.57	2	<0.0001
Antibiotics	911(47.1)	536(54.4)	1447(49.6)	13.828	2	0.001

An examination of the drug pressure with ACT, quinine and SP reveals the prescription practice is focused where the disease burden is highest in the less than 15 year old children. In the 2008 guidelines testing was not recommended or obliged for this group; however, at the Mbakong Health Centre some of them were tested. There was a significant association between age group and the prescription (Fig 2A) with a decreasing trend of ACTs with age.

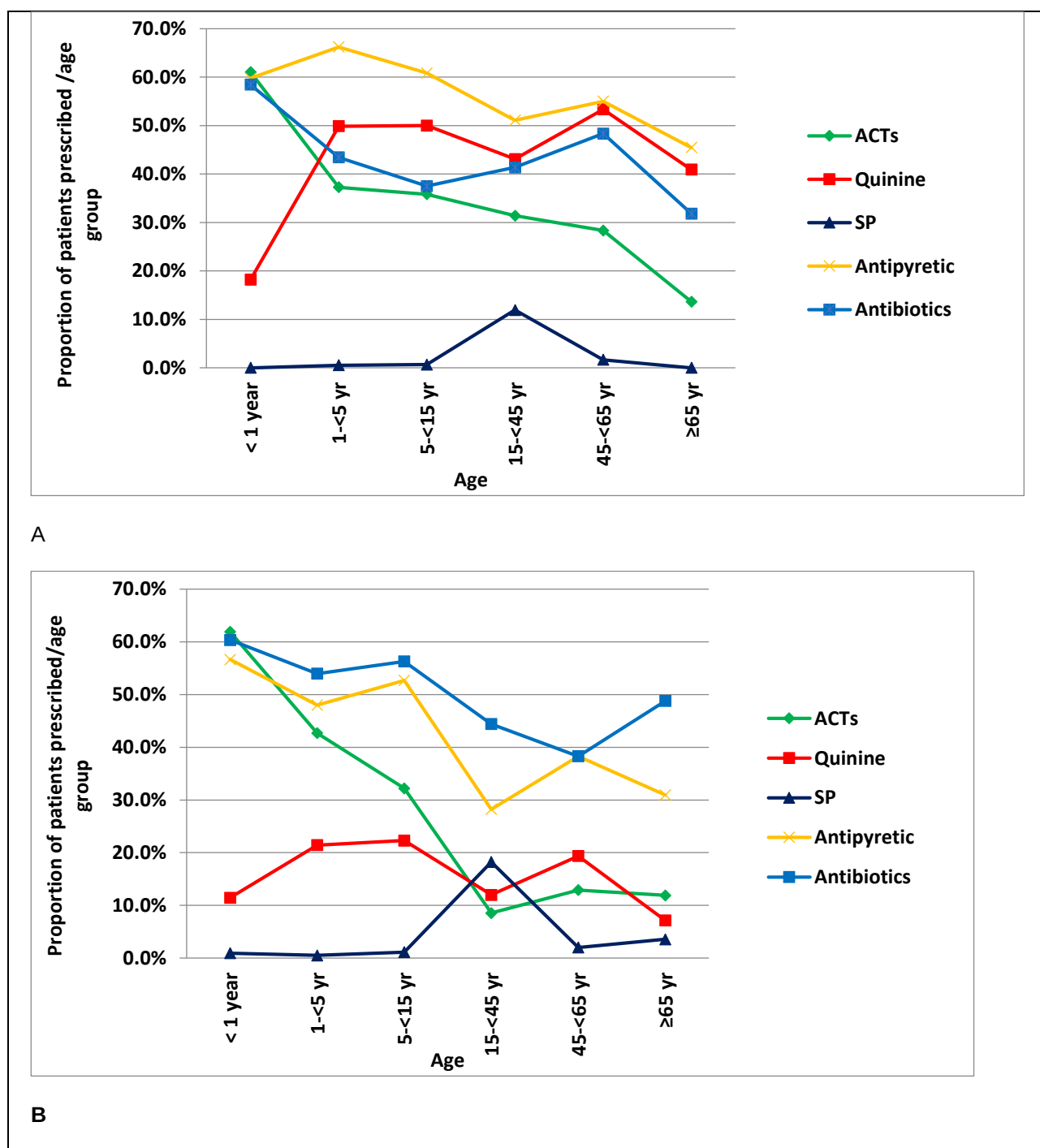


Fig 2: Pattern of treatment prescribed to febrile patients tested for malaria A) confirmed malaria positive patients. B) Confirmed malaria negative results. Each data point represents proportion of prescriptions of a particular drug regimen dispensed to a given age group during the period of the study.

In terms of numbers, the females who were prescribed therapy with confirmed malaria were about twice the number of males. However, the proportions of males who were prescribed ACTs and antipyretics were found to be significantly higher than the females across all age groups. The number of patients with negative results that were prescribed ACT, antibiotics and antipyretics were higher than those with confirmed results except in the case of quinine and SP where the numbers of confirmed malaria patients treated were higher than those treated with negative results.

3.3 Prescription to malaria positive patients

3.3.1 Severe malaria: Within the group of patients classified as suffering from severe malaria, 37 (13.7%) were confirmed to be malaria positive. Of these, 24 (64.86%) were prescribed quinine, 12 (32.43%) ACTs while 1 (2.70%) was prescribed SP. Concerning the distribution of severe malaria cases across age groups, the age groups that witnessed a relatively high number of severe malaria cases were fifteen to <45 years 14/37 (37.84%) followed by those of one to <5 and five to <15 years 8 (21.62%) respectively. There was no significant association between the treatment prescribed or received and the age groups ($p=0.37$) except for SP ($p=0.03$).

3.3.2 Uncomplicated malaria: Of the 1,202 patients clinically diagnosed to suffer from uncomplicated malaria and confirmed to be positive, 429 (35.69%) were prescribed ACTs, 542 (45.09%) quinine, 53 (4.41%) SP while 508 (42.26%) were prescribed an antibiotic. The prescription of ACTs was found to decrease with age with less than one year children being prescribed 63% while the ≥ 65 years received 14%. Quinine prescription was high in the age group forty five to <64 years with 53%, five to <15 years 50% and one to <5 years with 49%. The lowest level of Quinine prescription was observed in the less than one year old and those older than 65 years. There was a significant association between the prescriptions of ACT, quinine and SP with age group for febrile patients suffering from uncomplicated malaria that

were confirmed to be positive for malaria ($p < 0.001$). However, there was no significant association between the age groups with regards to the prescription of antibiotics ($p = 0.072$).

3.4 Prescriptions to patients with a negative test for malaria as per clinical diagnosis

3.4.1 Severe malaria: Of the 233 patients clinically diagnosed to suffer from severe malaria and that were confirmed to be malaria negative, 137 (58.8%) were treated with ACTs, 64 (27.5%) with quinine while 6 (2.6%) were treated with SP. The highest proportions of ACT were prescribed to children < 1 year 36 (76.6%) and one to < 5 years 48 (70.6%). A significant association was observed between the proportion of ACT prescribed and the different age groups (Pearson $X^2 = 24.992$, $p < 0.0001$). Under this classification, a total of 111 (47.6%) patients were prescribed antibiotics. Prescriptions of antibiotics was high in all age groups below 45 years but very low in the age group ≥ 65 years. However, there was no significant association between the prescription of SP, antibiotics (Pearson $X^2 = 13.49$, $p = 0.2011$) and antipyretics (Pearson $X^2 = .97$, $p = 0.29$) and age group.

3.4.2 Uncomplicated malaria: A total of 2,686 febrile cases that were clinically diagnosed to be suffering from uncomplicated malaria were treated for malaria despite being confirmed malaria negative. Of these, 609 (22.7%) were prescribed ACT, 401 (14.9%) quinine and 235 (8.7%) were prescribed SP (Table 3). One thousand three hundred and thirty six (49.7%) of these patients were prescribed antibiotics. ACT prescriptions were found to decrease with patient age with the < 1 year patients receiving 164 (59.4%) prescriptions while the ≥ 65 years patients received 9 (11.0%) prescriptions. Quinine prescription was found to be high in five to < 15 year old and one to < 5 year old patients with prescriptions of 83 (21.1%) and 112 (21.0%) respectively while the forty five to < 65 year olds patients received 42 (18.5%) prescriptions. A total of 1,336 (49.74) patients were prescribed antibiotics in this category. Antibiotics prescription was generally high in all age groups with a significant association between the drug prescribed and the age group (Pearson $X^2 = 62.92$, $p < 0.0001$).

Table 3: Treatment prescribed to those who were clinically diagnosed to suffer from uncomplicated malaria but were confirmed to be malaria negative

Number of prescriptions within the age (%)										
	< 1 year	1-<5yr	5-<15 yr	15-<45 yr	45-<65 yr	≥65 yr	total	Pearson χ^2	df	p-value
	n = 276	n = 254	n = 393	n = 1174	n = 227	n = 82	n = 2686			
ACTs	164(59.42)	209(39.14)	114(29.01)	89(7.58)	24(10.57)	9(10.98)	609(22.67)	484.364	10	<0.0001
Quinine	26(9.42)	112(20.97)	83(21.12)	133(11.33)	42(18.50)	5(6.1)	401(14.93)	57.594	10	<0.0001
SP	3(1.09)	2(0.37)	4(1.02)	219(18.65)	4(1.76)	3(3.66)	235(8.75)	281.804	10	<0.0001
Antipyretic	159(57.60)	245(45.88)	197(50.13)	321(27.34)	83(36.56)	24(29.27)	1029(38.31)	153.029	10	<0.0001
Antibiotics	173(62.68)	283(53.93)	222(56.49)	522(44.46)	90(39.65)	41(50.00)	1336(49.74)	62.924	10	<0.0001

The odds of being prescribed an anti-malarial were mostly higher in patients classified as suffering from severe malaria compared to simple malaria for ACT (OR=2.8; 95% CI 2012-3.65, $p<0.0001$), SP (OR=2.2, 95% CI 0.99-5.11, $p<0.04$) and for quinine (OR=1.00, 95% CI 0.74-1.47, $p<0.89$). Though this association was both statistically significant and practically visible for ACT it was not significant for quinine. The odds of being prescribed an antibiotic was higher in patients classified as suffering from simple malaria compared to severe malaria (OR=4.7, 95%CI 2.84-7.86, $p<0.0001$) with a practically significant association. The odds of being prescribed a combination of anti-malarials and antibiotics was high in patients classified to be suffering from severe malaria compared to patients suffering from simple malaria: ACT + Q (OR=6.2, 95%CI 2.35-16.48, $p=0.001$), ACT + AB (OR=1.9, 95% CI 1.43-2.54, $p<0.0001$), ACT + SP + AB (OR=6.6, 95% CI 0.60-73.45, $p<0.19$), Q + AB (OR=1.7, 95%CI 1.23-2.39, $p=0.002$). The odds ratios were practically significant for ACT + Q, the combination ACT + SP + AB was practically but not statistically significant while the combination Q + AB was not practically significant (Ellis and Steyn, 2003).

Concerning the number of returning cases, 40 (1.1%) patients of all those who were treated with anti-malarials returned to the health facility with a fever within one month. Of these, 17 (42.5%) were confirmed positive for malaria parasites (Ndong et al., 2014). It is not clear what the cause of this recrudescence was since no follow up study was conducted at this site. Also no study has been conducted to determine the parasite species as well as their susceptibility to the anti-malarials currently in use.

4. Discussion

The analysis revealed that uncomplicated malaria was clinically over-estimated by more than two thirds of the prevalence level while severe malaria was over-estimated by 88%. These results corroborated earlier reports of malaria over-diagnosis for febrile patients seeking treatment in Cameroon (Sayang et al., 2009; Mangham et al., 2012,) and were higher than those reported for severe malaria in Tanzania (Reyburn et al., 2004).

Anti-malarials were prescribed in different combinations and with antibiotics. Combinations such as ACT+AB, Q+AB, ACT+Q, ACT+Q or SP+Q+ACT reflect a continuation of the practice or a possible consequence of an incoherence with the treatment policy, where it was usual to include an antibiotic when treating for malaria as the absence of parasites in a blood film did not rule out malaria (Minsanté, 2008). In addition, combining two anti-malarials such as ACT and quinine of the same strength could be of potential danger to the patients.

The prescription practice revealed that the use of quinine fluctuates alternatively with the use of ACT. When quinine is high, ACT is low and vice versa. This could possibly mean that excessive use of quinine tends to adversely affect the use of ACT. The drug pressure was relatively higher in males for ACT and antibiotics while for females it was high for quinine and SP throughout the study period. These fluctuations in the prescription of quinine and ACT may point to the fact that healthcare givers are not fully detached from quinine use and would need more training or a modification in the policy to help change this practice. Incidentally, the

drastic increase in the prescription of ACT and drop in quinine was observed at a time (2008-2009) when the new guidelines were just made available to health facilities across the country following a series of trainings. This seems to have resulted in a peak in ACT prescription shortly after training but may have been diluted as time went on. Thus, continuous refresher training on the ongoing policy and monitoring of drug use is necessary especially at the Health centre level. It could be argued that the fluctuations observed between quinine and ACT could have been due to stock outs. Obviously, this is not the case as the North West Special Fund for Health conducts drug distribution to all public health facilities every fourth night as well as on special demand.

In the case of SP, there was a considerable refrain from its use. SP prescription maintained a decreasing pattern over time with more than 3/4 being prescribed to women of childbearing age. This shows a gradual adaptation to the new policy. Despite a consistent change in the prescription of SP, the fact that it was prescribed to febrile women of childbearing age still reflects an inappropriate scenario as it is reserved for asymptomatic pregnant women.

Over-treatment was observed across all the anti-malarial drug regimens. Close to two-thirds of the patients who were prescribed an ACT either had a negative result or were treated without a malaria test. This finding is consistent with (Mangham et al., 2012). Overuse of ACT in this manner poses a major problem in health service planning and resource allocation as ASAQ is subsidised by the government. In the 2008 malaria treatment guidelines quinine prescription was and is still reserved for severe malaria. Following an observed drop in the number of severe malaria cases, it was expected that over time the use of quinine should decline. This was, however, not the case as quinine was largely overused in treating uncomplicated malaria as well as confirmed malaria negative cases. Such practice does not only unnecessarily increase the cost of treatment but causes the patient to go through unnecessary pain resulting from the drug side effects. It is not clear from the data whether the patients' choices were

taken into consideration as it has been reported that patients do prefer certain treatments to others (Mangham et al., 2012, Mbacham et al., 2014).

Though the prescription of anti-malarials at this health centre suggests non-adherence to the new treatment policy, the policy itself has an ambiguity where it states that a negative result does not rule out malaria (Mangham et al., 2012, NMCP, 2008). In this light, the overuse of antibiotics seems to reflect a continuation of the old practices where antibiotics were associated with anti-malarials when treating febrile patients. Since the health worker is expected to play a key role in determining what services a patient receives, it is important to accept that the ability of the patient to pay for such services is crucial. At times, among other reasons, patients who cannot afford the cost of a test prefer to use the little money they have to pay for treatment. This inability of the patients to pay for a test also pushes some health workers to prescribe presumptively. However, at Mbakong Health Centre the proportion of patients treated without a test is relatively low. This suggests that the problem of over-treatment does not result from lack of testing services but rather to the degree of trust given the test results as earlier reported (Ansah et al., 2010, Chandler et al., 2012, Mangham et al., 2012, Mbacham et al., 2014, Mbonye et al., 2013).

The high proportions of ACT prescribed to the less than one year old children despite a lower malaria prevalence compared to the other age groups reflects the role played by presumptive treatment. This finding is in line with the report of (Mbonye et al., 2013). Although children below age 15 received the highest proportions of ACTs and quinine, the quinine pressure was low in children less than one year. The observation that the ACT pressure decreases with increase in age corroborates earlier findings by Sayang *et al*, (2009) that health workers preferably would prescribe amodiaquine (or an ACT in the light of the new policy) to children and quinine to adults. Thus, it could have been easy for the health workers to accept the transition from amodiaquine to ACTs for children than from quinine to the recommended ACTs

for adults. This view fits in with the prescriptions in the less than one year old group, however, it does not seem to hold for the one to <5 year old children as they have the highest quinine pressure.

Adherence to the test results is demonstrably a very serious problem in the study area. This is supported by the finding that more than half the patients with negative results were prescribed an ACT and more than 1/3 were prescribed quinine. Poor adherence to test results has been reported to be 45% in Cameroon (Mangham et al., 2012), 50% in Ghana (Ansah et al., 2010) and 54% in Tanzania (Reyburn et al., 2007). In line with the report of (Reyburn et al., 2007) the proportion of antibiotics prescribed was high in patients with negative results than those with positive results.

Apart from non-adherence to test results (Ansah et al., 2010), the general non respect for guidelines recommendations following clinical conditions remains a serious problem. This is supported by the observation that though the treatment policy required that quinine be used for severe malaria cases only, 1/3 of patients who were supposed to receive quinine were given an ACT. This practice contrasted sharply with the recommendations of the guidelines.

Though malaria over-treatment has already been well documented in Cameroon (Mangham et al., 2012), the results of this study zoom in on what takes place at the health centre level. This could reflect in miniature what goes on in other health facilities around the country. A scale up of this study is required to enable brought based conclusions. Given that the causes of most of the fevers in the health centre are not yet identified we suggest that an investigation be conducted to determine this.

5. Limitations of this study

The conclusions drawn from this study are based on case management records of febrile patients clinically diagnosed with malaria considered retrospectively over a seven-year period (2006–

2012) at the Mbakong Health Centre. Inclusion of more health facilities in a further study could improve on the results. This study did not follow up cases and therefore it is not possible to make valid conclusions on the returning cases. Also there were no records of the *Plasmodium* species in this area as well as their susceptibility to the anti-malarials in use.

6. Conclusion

Overall, there is over-diagnoses and over-treatment of malaria, thus departing from the WHO guidelines. Although there is an overall increase in the prescription of ACT and less prescription of quinine, the old practice is still rampant. A noticeable restrain from prescription of SP to febrile cases was observed. There is need for healthcare workers to adhere to guidelines in an attempt to enhance the rational use of drugs to achieve appropriate treatment of especially uncomplicated malaria. The results of this study act as a baseline for further studies in Cameroon and should be particularly useful to the National Malaria Control Programme.

7. Competing interests

The authors declare that they have no competing interests.

8. Authors' contribution

ICN: Conceived the study and experimental design, data collection, analysis and interpretation, preparation of the manuscript. WFM: Contributed substantially to conception, study design, interpretation of data, and critically reading the manuscript. AFG: Contributed to designing the molecular analysis and interpretation of the data as well as critically revising the manuscript. DAB: Experimental design of the study, analysis and critically revised manuscript for intellectual content. RvM: did the statistical analysis as well as critically reviewed the manuscript for intellectual content. All authors read and approved the manuscript.

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CHAPTER FIVE

KNOCK DOWN RESISTANCE PROFILE OF *ANOPHELES GAMBIAE* AND *ANOPHELES COLUZZII* IN MBAKONG, NORTH WEST REGION, CAMEROON

(MANUSCRIPT 1)

To be submitted to Acta Tropica Journal

Knock down resistance profile of *Anopheles gambiae* and *Anopheles coluzzii* in Mbakong,
North West Region, Cameroon

Ignatius C Ndong^{*a,b,c}, Daniel A Boakye^{*d}, Wilfred F Mbacham^{b,c} Anne F Grobler^a

a) *DST/NWU, Preclinical Drug Development Platform, North-West University, Potchefstroom
Campus, South Africa*

b) *Catholic University of Cameroon, Bamenda*

c) *Laboratory of Public Health Research Biotechnologies University of Yaoundé I, Cameroon,*

d) *Noguchi Memorial Institute of Medical Research, University of Ghana Legon.*

E-mail:

*Corresponding author:

Ignatius C Ndong: ndongicheng@yahoo.com, 25044672@nwu.ac.za,

Daniel A Boakye: dboakye@noguchi.mimcom.org

Wilfred F Mbacham: wfmbacham@yahoo.com

Anne F Grobler: anne.grobler@nwu.ac.za

Abstract

The government of Cameroon has in the past decade intensified efforts to control malaria through promoting the use of insecticide treated nets in an area where insecticide resistance is increasingly becoming a problem. The recent upsurge in the frequencies of knockdown resistance (*kdr*) could undermine these efforts. We investigated *kdr* profile of *Anopheles gambiae* and *An. coluzzii* in Mbakong, North West Region, Cameroon. Mosquitoes were collected using human volunteer. Following morphologically identification, PCR-based methods were used to identify sibling species of *An. gambiae* s.l, molecular species and to determine the presence of *kdr* mutations. Analyses were conducted using Microsoft Excell. The frequency of L1014F mutations in both molecular species specimens analysed was 41.18%. The frequency of this mutation was 41.67% in *An. gambiae* and 50.00% in *An. coluzzii* specimens. All three genotypes homozygous resistant, heterozygous and homozygous susceptible were detected in *An. gambiae* with frequencies of 2.08%, 20.83% and 2.70% respectively. In *An. coluzzii* only the homozygote susceptible and heterozygous genotypes were detected at frequencies of 35.00% and 15.00% respectively. The frequency of the L1014S mutations was 31% within the sampled population with 31% within *An. coluzzii* and 24% within *An. gambiae*. The frequencies of both the heterozygous and susceptible genotypes were 11% each while the homozygous genotype was 2% in *An. gambiae* samples. The frequency of the L1014S mutation in *An. coluzzii* was 19% in the homozygous susceptible state and 12% in the heterozygous state. Both the L1014F and L1014S mutations were found to co-occur in the same mosquito. The *kdr* mutations are widespread in Mbakong with higher frequencies in *Anopheles coluzzii* than in *Anopheles gambiae*. It is not clear what the impact of such a high frequency of *kdr* alleles in this area could be on the malaria transmission dynamics. There is need for further studies and close monitoring of the insecticide resistance status as well as the resistance mechanisms in this area. We intend to increase the sample size and improve on the statistical significance and relevance required for the publication of this study.

Key word: Knock down resistance, profile, mutation, *Anopheles gambiae*, *Anopheles coluzzii*, Mbakong, Cameroon

1. Background

Vector control is an important strategy in malaria control. Pyrethroid-impregnated bed nets are a central component of the World Health Organization's Global Strategy for Malaria Control (WHO, 1993a). The use of insecticide for malaria vector control holds particular promise in Africa where 80–90% of the world's malaria cases occur. However, the upsurge of resistance in the mosquito vectors to pyrethroids which are considered to be more effective with little side effect on human has the potential to undermine contributions already made by control efforts (Ranson et al., 2011). Across Africa insecticide resistance in malaria vectors has become a major concern for public health authorities and national malaria control programmes (Chouaïbou et al., 2008, Martinez-Torres et al., 1998, WHO, 2002). The Cameroon National Malaria Control Programme (NMCP) has in the past decade intensified efforts to prevent malaria through promoting the use of insecticide treated nets to reduce man-vector contact. Earlier on, the NMCP targeted only pregnant women and children less than five years. However, in 2012 more than eight million long last insecticidal nets (LLIN)/insecticide treated nets (ITN) were distributed nationwide to the general population. The insecticides used in treating the recently distributed LLIN/ITN in the North West Region were Deltamethrin and Lambdacyalothrin. This was aimed at scaling up the control and prevention efforts. In order to minimised widespread development of resistance to insecticides, the government of Cameroon has divided the country into seven malaria epidemiological strata or zones with alternating use of insecticides. The North West Region falls within zone V of the malaria epidemiologic map of

Cameroon (WHO, 2002) (Figure 1).

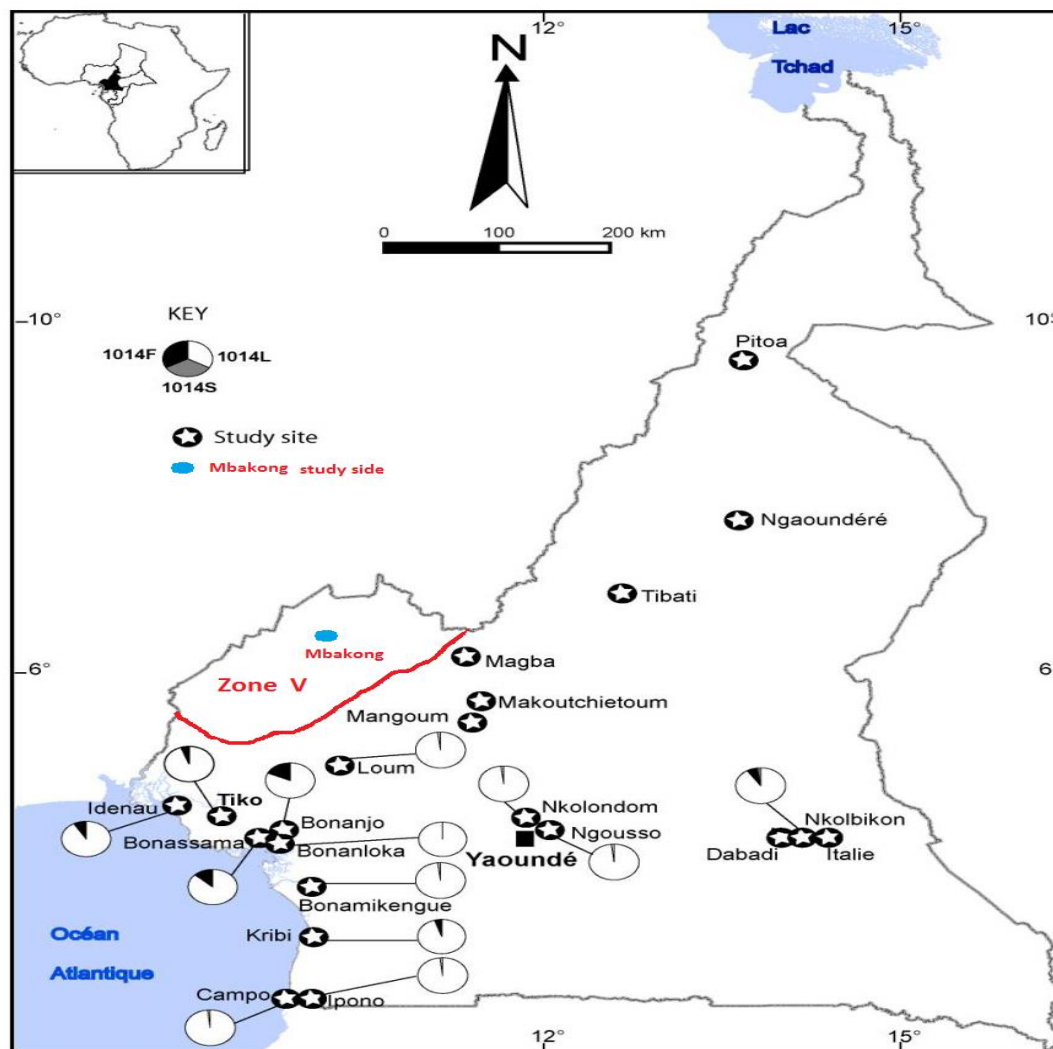


Figure 1: Map showing the Malaria Epidemic Zone five and the study site. Reproduced with permission.

Resistance to insecticides in the malaria vector can be the result of an alternative point mutation at position 1014 in the voltage-gated sodium channel resulting in the leucine-phenylalanine (L1014F) or a leucine-serine (L1014S) substitution termed the knock down resistant (*Kdr*) alleles or the over production of the detoxifying enzymes e.g. glutathione S-transferase, mono-oxygenase or esterase (Beaty and Marquardt, 1996, Santolamazza et al., 2008a). Extensive work has been conducted on

insecticide resistance in Cameroon especially within the last decade (Bigoga et al., 2007a, Chouaïbou et al., 2008, Etang et al., 2003, Reimer et al., 2008b, Wondji et al., 2005, Wondji et al., 2011, Nwane et al., 2009). In their report, Etang and colleagues (Etang et al., 2003) demonstrated that within the *An. gambiae* complex, *An. arabiensis*, *An. coluzzii* and *An. gambiae* molecular species had developed insecticide resistance. Owing to earlier reports that the distribution of *Kdr* alleles in West Africa had previously provided evidence of strong barrier to gene flow between *An. coluzzii* and *An.gambiae* (Chandre et al., 1999), Etang and colleagues tested specimens for 'L1014F' *Kdr* mutation.

Although DDT and dieldrin had once been used in this area in the 1950s for seven years (Cavaiié and Mouchet, 1962), no *Kdr* mutation (L1014F) were detected, leading to the conclusion that other mechanisms of insecticide such as the 'L1014S' mutation or detoxification enzymes may be at work to account for the resistance observed in the *An. coluzzii* and *An. gambiae* in the Northern rice fields of Cameroon. Two years later, *Kdr* mutations were detected in Campo in a heterozygous state (Etang et al., 2006). To compound this picture, in 2008 low level of *Kdr* mutations were reported in the northern cotton field (Chouaïbou et al., 2008). In a similar study Reimer *et al.*, (2008) had demonstrated the absence of *Kdr* in *An. coluzzii* and *An. gambiae* in Tiko, though, it was found in higher frequencies in both species in Bioko, Equatorial Guinea.

In a recent multi country study, Santolamazza *et al.*, (2011) reported that both *kdr* mutations – L1014F and L1014S are present and widely distributed in *An. gambiae* while only the L1014S mutation was reported in *An. coluzzii*. The L104S mutation in *An. coluzzii* was reported to be restricted to the coast of the Gulf of Guinea – Cameroon, Nigeria and Benin. These mutations have recently been reported to be widespread in the central Africa region and have been identified in Gabon, Equatorial Guinea, Angola, Chad and Cameroon (Etang et al., 2007, Etang et al., 2009a, Pinto et al., 2006, Pinto et al., 2007 , Kera-Hinzoumbé et al., 2008). In Cameroon both L1014F and L1014S mutation have been reported to occur in the same mosquito (Reimer et al., 2008b). Their co-

occurrence and rise in frequency in mosquito vector populations in Cameroon is suggested to be evidence of the ongoing geographical spread of both the L1014F and L1014S mutations invading wild *An. gambiae* populations throughout Africa (Nwane et al., 2011).

In a recent study to map the entomological resistance profile of various malaria vectors in Cameroon by the Ministry of Public Health, it was revealed that there is ongoing resistance development to insecticides such as DDT, Deltamethrin, permethrin, Lambdacyalothrin, and bendiocarb (Minsanté, 2010). These resistance species are sparsely distributed across different ecotypes and render control strategies an uphill task to planners. Apart from the insecticide pressure from the agro industrial concerns, domestic use of insecticide especially on bed nets has been ongoing for about ten years and could also be contributing to the development of the *kdr* mutation in the country (Nwane et al., 2011). According to the maps presented, there seem to be resistance to all the insecticides listed in the North West Region of the country where not many studies have been carried out including the Mbakong Health Area in the Bafut Health District.

This study investigated the distribution of L1014F and L1014S in Mbakong health area. The study site is located along the Mezam and Menchum Rivers near the Menchum fall that has been earmarked for the generation of hydro-electricity. An in-depth understanding of the type of *kdr* mutations and distribution in this area is not only important to the local population, but also to the NMCP to enable it improve its planning strategy for scaling up of malaria control programmes as well as the workers on the dam or tourists visiting the Menchum fall. The results of this study would serve as baseline for entomological studies in this area.

2. Methods

2.1 Study Area

Mbakong, with GPS coordinates 6°10'18.87" N and 10°05.40" E is a rural area located in the Bafut Heath District of the North West Region of Cameroon. The population of Mbakong health area is about 8,000 people. It is a riverine community along the Mezam and the Menchum rivers, which lies within the Guinea Savannah region at the border of the Bafut forest to the South and the Bu forest to the north. It experiences a maximum rainfall pattern in August with a mean annual rainfall between 0 and 390 mm and a monthly temperature range of 22.49-26.34°C. The dry season runs from November to March. Human activities in this area include farming (maize and rice), game hunting and sand mining from the Rivers. Sand mining is mainly carried out by young men (above 15 years). Malaria transmission in Mbakong is all year round with a prevalence of 29.30% among febrile patients seeking treatment (Ndong et al., 2014).

2.2 Sample Collection

Mosquitoes were collected using the Human Landing Catches (HLC) methods as described by WHO (WHO, 1975). Six collectors were recruited and trained after obtaining informed consent. Mosquito collection was done at three locations that were about 500m apart in the community, with two people per location. These locations were close-by to facilitate supervision. One person collected from 18:00-24:00 and the next person collected from 24:00-6:00 while the supervisor shuttled between locations to ensure that collectors did not sleep. This was done every fortnightly from August through December 2012.

2.3 Species identification

Morphological identification was done using reference keys (Gilles and De Meillon, 1968, Gillies and Coetzee, 1987a) and stored dry in tubes containing silica gel. DNA was extracted using ZR Genomic DNA™ – Tissue MiniPrep kit supplied by Inqaba Biotech South Africa following the instructions of the

manufacturer. Extracted DNA was stored at -20°C until required for PCR. The PCR-RFLP was used to identify specimens down to the sibling level and molecular species level following the method of Fanello *et al.* (Favia *et al.*, 2001). Some specimens that were identified as either the *M* or the *S* form revealed multiple bands during digestion. These were excluded from further analysis. It is worth noting that the *M* and *S* molecular forms of the former *Anopheles gambiae* s.s. have been given full species status. The *M* form (*An. coluzzii* Coetzee & Wilkerson **sp. n.**) and the *S* form retained the nominotypical name (*An. gambiae* Giles)(WHO, 2010). In this, study we use molecular species to refer to both mosquito species.

2.4 *kdr* amplification

Detection of the L1014F and L1014S *kdr* mutation was based on the modified PCR diagnostic test developed by Martinez-Torres *et al.* (Martinez-Torres *et al.*, 1998) and Ranson *et al.* (Ranson *et al.*, 2000). Two separate PCR reactions were performed for each specimen, one to detect L1014F using primers Agd3 and Agd4, respectively, and another in which primer Agd3 was replaced by Agd5 to detect L1014S. Each reaction contained 1 mL of DNA, 1 PCR buffer (Promega), 2 mM of MgCl₂, 0.2mM of each deoxynucleotide, primers Agd1 and Agd2 at 0.1 mM, primers Agd3 (or Agd5) and Agd4 at 0.4 mM, and 1U of Taq DNA polymerase (KAPABiosystems, South Africa), in a total volume of 20 mL. The PCR cycling conditions were an initial step of 5 min at 94°C followed by 35 cycles each with 30s at 94°C, 40 s at 48°C (for Agd3) or 55°C (for Agd5) and 40s at 72°C, and a final 10-min extension at 72°C. For specimens that showed only the internal control band at 293bp, the annealing temperature of 55°C was varied by ±1°C to see whether it would change. This variation in temperature did not lead to any noticeable change. All PCR products were visualized using a BioRAD gel documentation system.

Name	Sequence	Tm
Agd1	ATAGATTCCCCGACCATG	57.62
Agd2	AGACAAGGATGATGAACC	55.34
Agd3	AATTTGCATTACTTACGACA	52.20
Agd4	CTGTAGTGATAGGAAATTTA	52.20
Agd5	TTTGCATTACTTACGACTG	53.69

2.5 Ethical issues

The ethical clearance for this work was obtained from the Institutional Review Board of the Catholic University of Cameroon, Bamenda. All the participants who accepted to take part in the sample collection signed the consent form.

3. Results

A total of 530 *Anopheles* mosquitoes were caught. Of these 498 (93.96%) mosquitoes were morphologically identified as *An. gambiae* s.l. while 32 (6.04%) were *An. funestus*. The peak biting hours of *An. gambiae* seeking a blood meal were observed to be 10-11pm and 3-4:00am. Though catching did not exceed 6:00am, the number of biting mosquitoes at this hour was quite high (Figure 2).

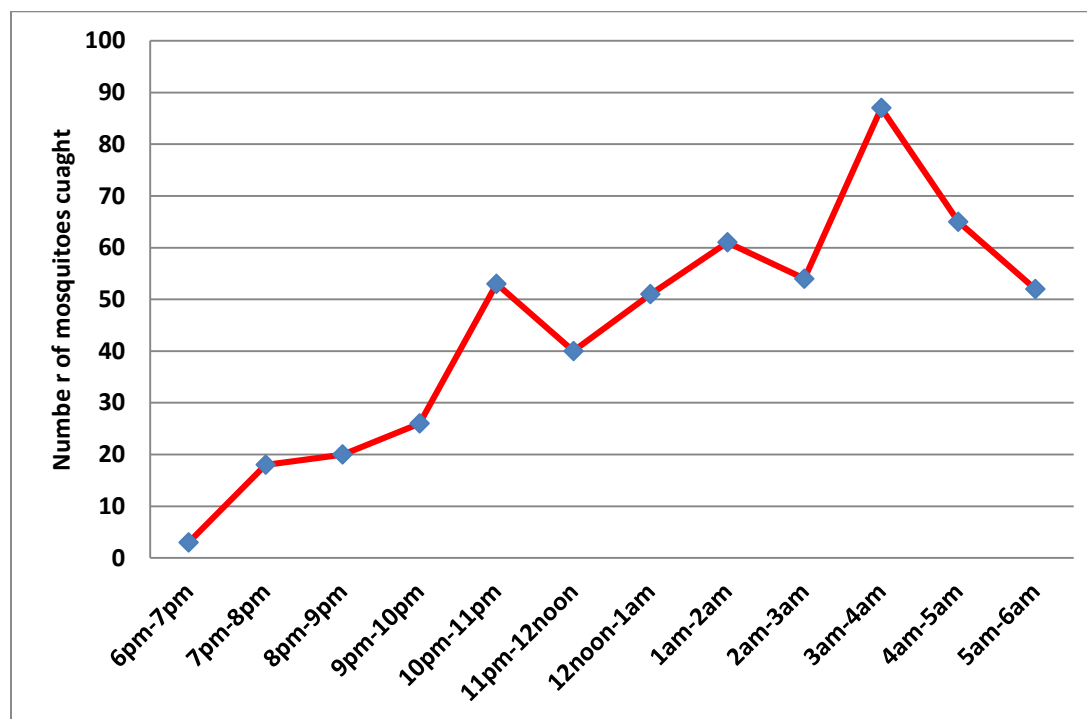


Figure 2: Hourly biting pattern of *Anopheline* mosquitoes by Human Landing Catches during the period August to December 2012.

Of these, 271/498 (54.42%) specimens were identified to the species level as *An. gambiae* s.l. while 33/498(6.63%) showed multiple bands. A total of 261 specimens including the 33 that showed multiple bands at the species level were subjected to digestion using Hha1 for molecular identification. Of these, 158/261 (60.54%) were identified to be *An. gambiae* while 70/261 (26.82%) were *An. coluzzii*. Within the 33 specimens that showed multiple bands, 19/261 (7.28%) revealed bands characteristic of *An. gambiae* while 8/261(3.07%) showed bands characteristic of *An. coluzzii* bands but the results of 6/261(2.30%) specimens were not conclusive (Table 2)

Table 2: Distribution of specimens identified to the molecular species level.

Molecular form Identity	Frequency	Percent
<i>An. gambiae</i>	158	60.54
<i>An. coluzzii</i>	70	26.82
<i>An. gambiae</i> S form with multiple bands	19	7.28
<i>An. gambiae</i> M form with multiple bands	8	3.07
Inconclusive results	6	2.30
Total	261	100

Sixty eight mosquito specimens (48 *An. gambiae* and 20 *An. coluzzii*) were diagnosed for the presence of L1014F mutation. Overall, 28/68 (41.18%) of the specimens were found to carry the L1014F mutation – 14/68 (20.59%) specimens carried the homozygous susceptible genotype, 13/68 (19.12%) the heterozygous and 1/68 (1.47%) specimen carried the homozygous resistant genotype. This was found to be consistent with Hardy Weinberg equilibrium ($X^2 = 2.71$, $p=0.10$). As per species, the frequency of the L1014F mutation was 20/48 (41.67%) within the *An. gambiae* specimens - 10/48 (20.83%) specimens carried the heterozygous genotype 1/48 (2.08%) the homozygous resistant genotype while 7/37(2.70%) carried homozygote susceptible genotype ($X^2 = 1.13$, $p=0.29$). Two specimens (4.17%) showed the internal control band at 293bp but had neither the 195bp nor the 137bp band to confirm the status of the specimen. The annealing temperature of 48°C was varied by $\pm 1^\circ\text{C}$ but this modification did not yield any results. One possible cause could be that the DNA was degraded. On the other hand the frequency of the L1014F was found to be 10/20(50%) within the *An. coluzzii* population sampled – 7/20(35.00%) specimens were found to carry the homozygous susceptible genotype and 3/20(15.00%) specimens carried the heterozygous genotype but no homozygous resistant mutation was observed ($X^2 = 0.31$, $p=0.57$). The result of 1/20 (5.00%) specimen was inconclusive as only the band at 293bp was detected (Figure 3).

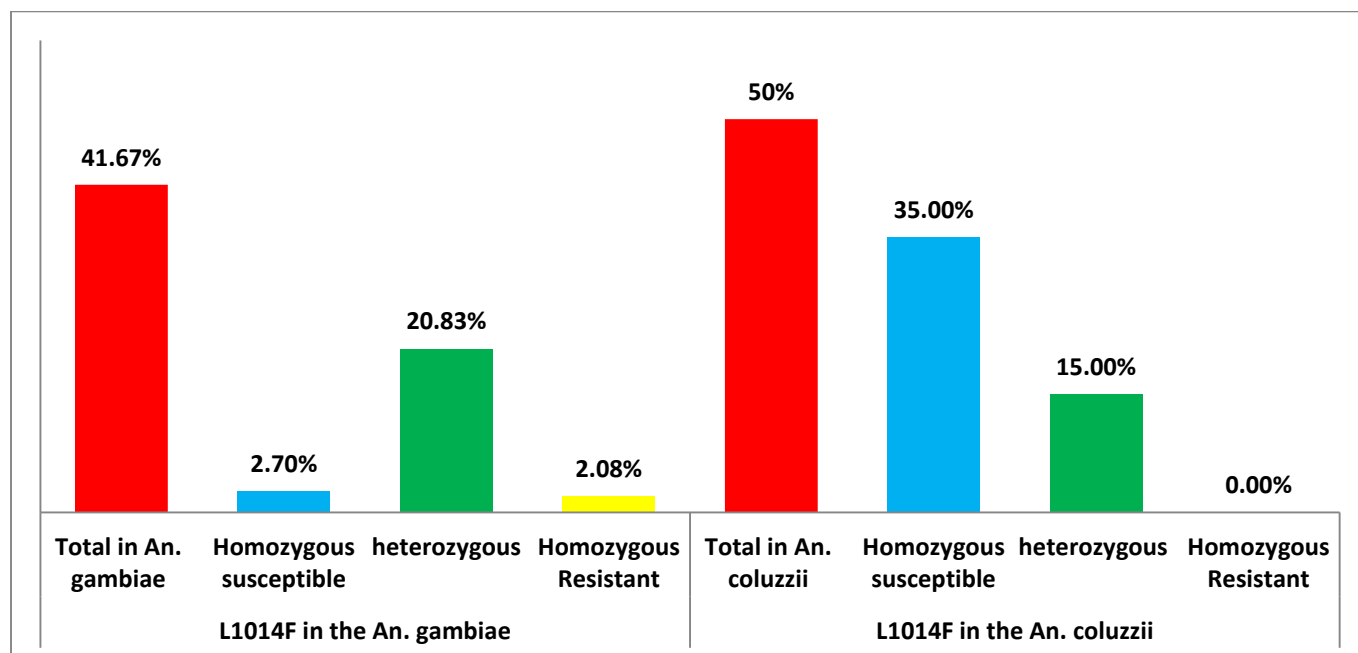


Figure 3: Distribution of L1014F mutation in both *An. gambiae* and *An. coluzzii*

A total of 71 mosquitoes specimens were assessed for the presence of the L1014S mutations- 45/71 (63.38%) being *An. gambiae* while 26/71 (32.91%) specimens were *An. coluzzii*. Overall, 22/71 (30.99%) of the specimens carried the L1014S mutation at varying frequencies – 10/71 (14.08%) specimens carried the homozygous susceptible genotype, 8/71 (10.13%) the heterozygous and 1/71 (1.41%) specimens was homozygous resistant. The genotype frequencies were found to be consistent with Hardy Weinberg equilibrium ($X^2 = 0.03$, $p=0.87$). This *kdr* mutation was absent in 14/71 (17.72%) of the mosquito specimens while 39 (54.93%) revealed inconclusive results showing only the internal control band at 293bp. It is not clear what the cause of this was. Varying the annealing temperature by 1°C did not change the results. This could have been due to degraded DNA but it could not be verified as specimens have not yet been sequenced. The frequency of L1014S was higher in the *An. coluzzii* 8/26 (30.77%) compared to *An. gambiae* 11/45 (24.44%). In *An. gambiae*, both the homozygote susceptible and heterozygote L1014S mutations were detected at a frequency of 5/45 (11.11%) each and the homozygote resistant mutation at a frequency of 1/45 (2.22%) ($X^2 = 0.84$, $p=0.36$). However, in *An. coluzzii*, only the homozygote susceptible and

heterozygote genotypes were detected at frequencies of 5/26 (19.23%) and 3/26 (11.54%) respectively ($X^2 = 0.26$, $p=0.61$) (Figure 4).

Interestingly analyses revealed that both *kdr* mutations occurred in the same mosquito. In the *An. gambiae* population sampled it was observed that the L1014F and L1014S co-existed in 3 specimens while in the *An. coluzzii* population the mutations were found to co-exist in 3 specimens. The number of samples analysed is very small to produce reliable results using the Hardy Weinberg equation. The next phase of this project would involve increasing the sample size to obtain statistically significant.

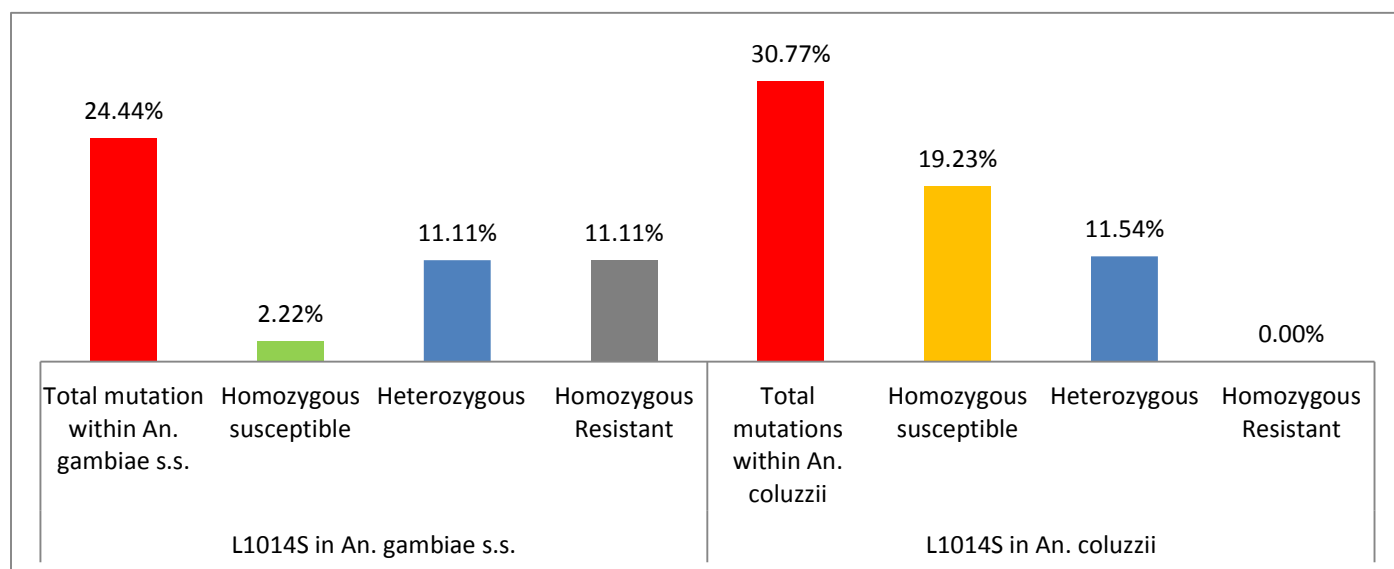


Figure 4: Distribution of L1014S mutation in both *An. gambiae* s.s. and *An. Coluzzii*

4. Discussion

Anopheles gambiae, *An. coluzzii* and *An. funestus* make up part of the mosquito fauna in Mbakong. The hourly biting pattern of the *Anopheles* mosquitoes in Mbakong revealed that the number of biting mosquitoes seeking a blood meal does not drop by 6:00am in the morning. This suggests that transmission could go on even among people who are going out very early for farming or those carrying out their early morning activities. This result corroborates earlier findings (Tchouassi et al., 2012). The results of this study revealed that the frequencies of L1014F *kdr* mutation is about 44% in the sampled population -with 50% occurring in *An. coluzzii* and 42% in *An. gambiae*. All three genotypes – homozygous susceptible, homozygous resistant and heterozygous were detected in *An. gambiae*. The findings of this study revealed that the frequencies of the L1014F mutations in both molecular species are high in Mbakong compared to the pre-2010 reports where the frequencies were very low in Cameroon (Chouaïbou et al., 2008, Etang et al., 2007, Nwane et al., 2009). Following a recent reports by Nwane and colleagues, the frequencies of the L1014F *kdr* mutations in *An. gambiae* in Mbakong are low when compared to those of the western highlands of 85% in Mangoum and 88% in Makoutchietoum (Nwane et al., 2011). The L1014F *kdr* mutation frequencies observed in the *An. coluzzii* are generally high compared to those obtained by Nwane and colleagues in the forest and coastal areas of Cameroon but lower than the 68% reported by (Antonio-Nkondjio et al., 2011) in Douala. Though in the study by (Nwane et al., 2011) the L1014F mutations were not reported in the Highland areas, the results of this study revealed high frequencies of these mutations in Mbakong, which is also a seasonal rice field. Two of the *An. gambiae* specimens (5%) had only the internal control bands at 293bp, indicative of the presence of the genotype but there was no additional band either at 195 or 137bp or both to provide additional information on the status, despite efforts to vary the temperature. It is not clear what the cause of this was. DNA degradation has been known to be at the source of abnormal results, it cannot be ascertained whether this was the case in

this study. Further sampling and sequencing of the present specimen could definitely throw light on the status of these specimens.

The L1014S mutations frequencies were 31% in the molecular species population sampled. These frequencies were distributed as follows - 24% in the *An. gambiae* and 31% in the *An. coluzzii*. In *An. gambiae* the frequencies of the homozygous genotype was 2% while those of the susceptible and heterozygous genotype were found to be 11% each. On the other hand, in *An. coluzzii*, homozygous susceptible genotype was found to be 19% and the heterozygous *kdr* mutations 12%. The frequencies of L1014S *kdr* alleles observed in this study were higher than those reported by Nwane and colleagues in *Anopheles gambiae*, 15% in Mangoum and 11% in Makoutchietoum (Nwane et al., 2011) as well as those reported by (Reimer et al., 2008b) 14% for Santchou and Bertoua. Though the L1014S was not detected in *Anopheles coluzzii* in the Western highlands as reported by Nwane et al., the frequency of 31% observed in our study is high. Also, it was observed that only the *An. gambiae* carried both the L1014F and L1014S *kdr* mutations in the homozygote resistant state.

The findings of this study clearly demonstrated that both L1014F and L1014S *kdr* mutations are present in the *An. coluzzii* and *An. gambiae* (Mbonye et al., 2013b); though higher in the *An. coluzzii* than *An. gambiae*. This observation is in contrast to earlier reports that L1014F mutation is more associated with *An. gambiae* in areas where both are sympatric (Awolola et al., 2003, Reimer et al., 2008b, Yawson et al., 2004).

However, the fact that the mutations are present in *An. coluzzii* and *An. gambiae* is consistent with the report of Remier et al., (2005). Furthermore, the mosquitoes have been found to carry both *kdr* alleles in the same mosquito in Santchou and Bertoua at lower frequencies (Reimer et al., 2008b). Mbakong is situated in a valley and is a subsistent rice cultivating community and the microclimatic conditions may be similar to those of Santchou. This area has been involved in subsistent rice cultivation for decades and it is not clear whether the use of insecticides for pest control could have

contributed to the *kdr* mutation frequencies observed in this study. There is need to investigate the resistance mechanisms in this area.

Our investigation did not look at the effect of phenotype on resistance and we limit out observation to the presence or absence of *kdr* mutations. Remier *et al* [10] had earlier observed that a majority of mosquitoes that were resistant to insecticides carried at least a copy of *kdr*. However, *kdr* alone does not confer complete resistance to the recommended pyrethroids used in the field. And that both L1014S and L1014F mutations are strongly associated with DDT resistance but weakly associated with type II pyrethroids. The observation that L1014F and L1014S are associated with resistance to insecticide (DDT and pyrethroids) currently in used, poses a serious problem to the current highly acclaimed indoor residual spray control strategy that uses DDT. However, there is a likelihood that changing to the type II pyrethroid in areas such as Mbakong could still produce the desired result (Ranson *et al.*, 2011). The LLIN distributed in this area are treated with Lambdacyalothrin (a type II pyrethroid) and deltamethrin. This shows that type II insecticides are already in use in this area. The distribution of the *Kdr* alleles in this area indicates the need to conduct further studies in the surrounding health areas to determine the *kdr* profile of two molecular species. This could help to enhance proper planning and adoption of appropriate strategies for insecticide use for vector control in the North West Region.

This high proportion of inconsistent results observed in this study could lead to an underestimation of the level of *kdr* in the Mbakong area. However, if the high frequencies of the *kdr* mutation observed reflect the degree of resistance to the presently used pyrethroids, then, this would be a very worrying picture for the national malaria control programme. This is because such could eventually weaken the observed decrease in prevalence that could be interpreted as impacts of interventions under the current malaria treatment policy. The spread of the *kdr*-resistant mosquito strains could facilitate the transmission of the malaria parasite, despite the use of LLIN as the biting period seems to extend beyond bedtime. This finding serves as a baseline for further studies in this area and the Region.

5. Limitations of the study

The conclusions drawn from this work are limited to the samples from Mbakong and so caution needs to be exercised when generalizing the findings. One other limitation of this work is that we have not been able to further investigate the actual identity of those specimens that showed multiple bands. We are considering increasing the number of samples as well as sequencing some of them. This is aimed at identifying the sequence variation in the specimens that produced multiple bands as well as those samples that showed only the band at 293bp. This would help throw light on the observations made in the course of this work as well as draw meaningful conclusion.

6. Conclusion

The *kdr* mutations (L1014F and L1014S) are widespread in Mbakong with higher frequencies in both *An.coluzzii* and *An. gambiae*. At the moment, it is not clear what the impact of such a high frequency of *kdr* mutation in this area could be on the malaria transmission dynamics. There is need for further studies and close monitoring of the insecticide resistance status as well as the resistance mechanisms in this area. We intend to increase the number of samples analysed in order to improve on the statistical significance in order to make reliable conclusion.

7. Competing Interests

The authors declare that they have no competing interests.

8. Authors' contribution

ICN: Conceived the study and experimental design, data collection, analysis and interpretation, preparation of the manuscript. WFM: Contributed substantially to conception, study design, interpretation of data, and critically reading the manuscript. AFG: Contributed to designing the molecular analysis and interpretation of the data as well as critically revising the manuscript. DAB:

Experimental design of the study, analysis and critically revised manuscript for intellectual content.
All authors read and approved the manuscript.

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Instruction for authors

(see page 140)

CHAPTER SIX

SUMMARY

6.1 Summary

This study largely focused on determining the trends in malaria admissions, case management and the knock down resistance profile of *Anopheles gambiae* s.s. in Mbakong, Cameroon, seven years following after changes in the national malaria treatment guidelines. Curve estimation – linear, quadratic and the cubic models – were used to check for trends in malaria admission over time.

Chapter three describes malaria admission trends over a seven year period, 2006-2012. Attempts to determine a trend in terms of years was not fruitful due to the limited number of data point. When the data were split into months a trend was found to be statistically significant in all models but only the cubic model explained sufficient variation to be of practical relevance ($R^2=0.571$). Furthermore, when the months were grouped into quarters of three months each, the cubic model also revealed a fluctuating and declining trends over time that was both statistically significant and practically visible ($R^2 =0.638$, $p<0.0001$)(Ellis and Steyn, 2003).

Malaria prevalence in Mbakong was found to be 29.3% corroborating earlier findings (Mangham et al., 2012, Ndong et al., 2014). In terms of seasonality, no seasonal trend could be established in the data. This is contrary to popular perception that there is a major transmission season during the rainy season between May and August and minor seasons between February to April and September to November. In addition, the perception of a December-January spike, in the heart of the dry season could be of interest since the mosquito population at this time is very low and consequently low malaria transmission (Antonio-Nkondjio et al., 2005, Bigoga et al., 2007b). However, these perceptions could not be verified in the data used for this study. There is need to investigate further malaria prevalence in asymptomatic individuals from December through January in this area.

Of all patients tested, males were found to have a slightly higher risk of testing positive for malaria than the females although females made up 2/3 of the malaria positive cases. Children younger than 15 years of age were found to be the hardest hit by malaria in the area corroborating earlier findings

(Mangham et al., 2012). Interestingly, children younger than 15 years represent the age group that could be used to monitor changes in prevalence and consequently a target group for mass drug administration (MDA). A practically visible and significant association was observed between testing positive for malaria, age group and gender (Pearson $X^2 = 153.675$, $p < 0.00001$, Cramer's $V = 0.352$).

Chapter four evaluated the management of malaria cases at the Mbakong Health Centre. Data analyses revealed that over-diagnosis of malaria and over-treatment due to non-adherence to test results and consequently non compliance to treatment guidelines were commonly practiced from 2006-2012. This was in line with earlier reports of over-prescription of anti-malarials in Ghana (Ansah et al., 2010b), Uganda (Kyabayinze et al., 2010), Kenya (NIS, 2012b), Tanzania (Ngasala et al., 2008), Zambia (Yeboah-Antwi et al., 2010), Nigeria (Mangham et al., 2011) and Cameroon (Mangham et al., 2012). Sixty one percent of ACTs were prescribed to malaria negative patients, 36.27% to malaria positive patients and 2.38% of patients were treated without a test. Fifty four percent of those patients treated with quinine were confirmed malaria positive, 44.54% negative and 1.23% of patients were treated without a test. Also, the use of quinine in the treatment of uncomplicated malaria seems to negatively affect the effective and full scale use of ACT as well as correct implementation of the guidelines. Though there is an overall improvement in the prescription of ACT and quinine the drug pressure is very high especially on the under 15 year old children. There seem to be good adherence of the health workers to the new policy in terms to SP prescription. The use of antibiotics in the treatment of febrile patients was generally very high compared to the number of patients confirmed to be suffering from bacterial infections. More females (63%) were treated with antibiotics than the males (37%).

Chapter five focused on the *kdr* mutation status of *Anopheles gambiae* and *An. coluzzii*. *Anopheles gambiae* s.s. specimens were morphologically and molecular identified and then genotyped using PCR. The results revealed that *kdr* mutations – L1014F and L1014S - are present in both molecular species at high frequencies in Mbakong. The frequencies of both mutations are higher in *An. coluzzii*

than in *An. gambiae*. While the susceptible, homozygous resistant and heterozygous genotypes were detected in the *An. gambiae*, the susceptible genotype were not detected in the *An. coluzzii*. The presence of both mutations in the mosquito populations in Mbakong could be a warning sign that the *kdr* mutations are widespread in this and surrounding areas. This paints a worrying picture to the national malaria control programme as it would require if need be switching from type I pyrethroid to type II since both mutations are known to be associated with resistance to DDT and type I pyrethroids. This would pose a serious problem to indoor residual spraying (IRS) using DDT. It is important that urgent measures be taken without which the success recorded with other control interventions under the current policy could be compromised should the *kdr* resistant alleles spread further in the mosquito population. If such mutations spread the presently observed low prevalence level of malaria could be reversed.

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CHAPTER SEVEN

FUTURE PROSPECTS AND RECOMMENDATIONS.

7.1 Extend study and increase the number of participating facilities

It is important to extend this study to other facilities and communities to obtain a broader picture. There is a serious need to investigate the causes of the non malaria febrile cases registered in this area (Ndong et al., 2014). There could be infections that are not detected by microscopy which are handled by the antibiotics prescribed and so these infections never get to be identified. Identifying these infections could help improve the care offered the local populations in this area. Developing multiplex diagnostic tools that could test for more than one disease could provide a solution to the problem of diagnosis currently faces in the field.

7.2 Need to conduct further studies on the mechanism of resistance in this area

A thorough understanding of the resistance mechanism in this area would help the national malaria control programme in planning and implementing vector control interventions especially in rural areas. Mbakong has a huge touristic potential as it is close the Mechum fall. Therefore, understanding the mechanisms of resistance is of special interest to the tourism industry that is fast expanding in Cameroon.

7.3 Need for closer monitoring of the insecticide resistance status in the area.

Monitoring of the *kdr* mutation spread in this area could inform the government on when to switch insecticides in order to avoid development of resistance to the insecticides in use.

7.4 Control efforts need to be stepped up.

Apart from chemotherapy, the distribution of LLIN should be followed by continuous community education programmes that encourage and motivate use.

7.5 Establish monitoring scheme with specific reference to vulnerable groups.

Mapping the vulnerable groups in this and other areas using prevalence data could be important in planning targeted mass drug administration (MDA). Targeted MDA implementation could take place when malaria prevalence is observed to be on the rise in a particular area within a given age group. This could be possible if local epidemiologic maps are developed using the complete hospital data as implored in this study. For instance the 2011 up surge in malaria in Mbakong was observed mainly in the age group 1-<15 year. An MDA campaign could target children within this age group during a high transmission when prevalence trends of incoming data within a given season predict a possible rise in a properly monitored health system.

7.6 Step up health workers' sensitisation programmes to reinforce policy implementation.

There is need for refresher and continuous training of health workers at the level of the health facilities. This could constantly update health worker knowledge, empower them to appreciate the need for changes in guidelines, enhance adherence to guidelines as well as appropriate use of subsidised medicine (Mbacham et al., 2014, Achonduh et al., 2014). Finally, the time lapse between the introduction of one guideline and then next should be extended.

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ANNEXURES

ANNEXSURE 1

Ethical Clearance

CATHOLIC UNIVERSITY OF CAMEROON,
BOX 872, MANKON, BAMENDA



Institutional Review Board

ETHICAL CLEARANCE

NDONG IGNATIUS CHENG

The Health Economics Policy and Management Department

Ethics Reference No: 001/HEPM/CATUC-IRB/17

Please quote this ref on all correspondence

Project Title: Characterising Malaria Prevalence/Management Trends and Transmission Competence of *Anopheles gambiae* s.s. in Mbakong, North West Region of Cameroon following changes in Malaria Treatment Policy 2006-2012

Investigator(s) Name(s)

1. Ndong Ignatius Cheng

Dear Ndong Ignatius Cheng

Your research protocol entitled **Characterising Malaria Prevalence/Management Trends and Transmission Competence of *Anopheles gambiae* s.s. in Mbakong, North West Region of Cameroon following changes in Malaria Treatment Policy 2006-2012** has this day been reviewed by the a joint institutional review board of the Biotechnology Center of the University of Yaoundé I and the Catholic University of Cameroon in Bamenda with the following remarks:

- Site(s) of Study: The researcher in the protocol should be specific of the geographical site(s) by GPS coordinates
- Further simplify the language of the Informed Consent

Therefore, the chair is satisfied with the decision of the committee and agreed to that there is no objection on ethical grounds to the proposed study. The study is of high medical and socio-economic importance; its implementation would contribute significantly to understanding health challenges. Adequate precautions have been taken by the researcher to manage potential risks that may arise in the course of this study. The IRB is therefore pleased to grant approval on the understanding that the research team will strictly abide to the conditions of the approval below:

- Strictly follow the plan of the approved protocol. Any changes to the approved protocol will require prior IRB approval.
- Must be promptly reported to the IRB: 1) Any deviations from or changes to the protocol which are made to eliminate immediate hazards to the study participant; 2) All risks that may be rare or remote and especially those which may entail serious consequences or compromise potential benefits or that would affect the conduct of the research.
- Must prepare and submit a standard progress report of the research study to the IRB at completion or one year from the date of issue of the approval letter and thereafter, on an annual basis.
- Must notify the IRB when the research is completed. Failure to submit an annual progress report on the study may affect the conditions of approval.

Your approval is given for one year renewable, on condition that a progress report is submitted to the IRB. While the has given its approval for this study on a satisfactory ethical basis, it is still necessary for you to obtain management approval from the relevant clinical directors or the health administrations where the work will be carried out

Sincerely,

Wilfred Mbacham, MS, DS, MPH, ScD, FAS, FCAS

Chair: CATUC IRB

Chair HEPM CATUC

Associate Professor of Public Health Biotechnology

Dr Tange Emmanuel

Co-Chair CATUC IRB

Provost School of Agricultural Sciences

Senior lecturer in Agricultural Sciences



Fides et Ratio, Sedes Sapientiae

ANNEXSURE 2

Conference participation

Oral Presentation L27

MIM Conference 2013.Durban South Africa

L27: Trends in malaria and malaria associated disease prevalence and treatment following changes in malaria treatment policy in the Mbakong health area of the North West region of Cameroon**Ndong I Cheng^{1,2,3}, Anne F Grobler¹, Daniel A Boakye⁴, Wilfred F Mbacham^{2,3}**

¹DST/NWU, Preclinical Drug Development Platform, Potchefstroom Campus South Africa; ²Department of Health Economic Policy and Management, School of Health Sciences, Catholic University of Cameroon, Bamenda; ³Laboratory of Public Health Research Biotechnology, Nkolbisson, University of Yaounde I, Cameroon; ⁴Noguchi Memorial Institute of Medical Research, University of Ghana, Legon.

BACKGROUND: There is an urgent need to assess scaled up control efforts requiring a deeper understanding of malaria associated diseases, which are most of the times neglected. Despite the reported reduction in malaria prevalence, febrile illnesses are often treated as malaria. We investigated the prevalence of malaria as well as the case management pattern of febrile patients over a seven year period (2006-2012) in a malaria endemic area in Cameroon, following changes in the treatment policy to ACTs.

METHODS: Data was collected during a survey consisting of consultation and perusal of laboratory and prescription registers of the Mbakong Health Centre in the Bafut Health District of Cameroon. Data analysis was conducted using SPSS Statistics.

RESULTS: Analysis revealed that 3562 febrile patients were treated from 2006-2012. Of these 995 (27.934%) were confirmed malaria positive with a monthly mean of 83 individuals and a monthly case negative mean of 214 individuals. Over the years confirmed malaria positive cases dropped steadily from 49.632% in 2006 to 11.433% in 2011 and then rose to 18.856% in 2012.

Disparity was observed between the proportion of confirmed malaria cases and the number of patients with the proportion of negative cases treated for malaria increasing from 15.566% in 2006 to 60.466% in 2009 and then a drop again to 12.500% in 2012. Over the study period 33.043% of patients were over treated for malaria.

ACTs, quinine and antibiotics were used in managing uncomplicated malaria. The mean annual drug pressure (*Number. of patients treated with a particular drug regimen*) from 2006-2012 was found to be 170 for ACT, 136 for quinine and 161 for Antibiotics. Children aged 1-4 and women 15-44 received the highest drug pressure.

DISCUSSION AND CONCLUSION: This study demonstrates that while the prevalence of malaria in Mbakong is decreasing, there does not seem to be a concomitant change in the prevalence of associated fevers. Malaria emerges therefore as not being the cause of all the fevers and calls for further investigation into non-malaria fevers. Malaria is over diagnosed and a high inappropriate use of quinine (third line drug) and antibiotics in managing uncomplicated malaria.

Guidelines International Network Conference 2014. Melbourne - Australia

Parallel Session 3.2: Oral presentations**Putting dollars to treatments varying from guidelines for malaria treatment in Cameroon: An economic evaluation**

Ndong IGNATIUS CHENG^{1,2,3}, Mari VAN REENEN⁶, Mbah Patrick OKWEN^{2,5}, Daniel A BOAKYE⁴, Anne F GROBLER¹, Wilfred F MBACHAM^{2,3},

¹ DST/NWU, Preclinical Drug Development Platform, South Africa

² Catholic University of Cameroon, Cameroon

³ Laboratory of PH Research Biotechnology, University of Yaounde I, Cameroon

⁴ Noguchi Memorial Institute of Medical Research, University of Ghana Legon, Ghana

⁵ Centre For the Development of Best Practices in Health, Cameroon

⁶ North West University Statistical Consultancy Service

Background: It is urgent to assess economic burden due to non-adherence to guidelines for malaria treatment. Despite reported reduction in malaria prevalence, malaria associated febrile illnesses are increasing in Cameroon and often treated as malaria. We investigate malaria prevalence versus case management pattern of febrile patients from 2006-2012 with a piggyback economic evaluation in a malaria endemic area in Cameroon, following changes in treatment guidelines.

Methods: Data was collected from hospital records at Mbakong Health-Centre, Cameroon and analysed using SPSS. We extrapolated the cost of inappropriate treatment from primary healthcare units in Cameroon.

Results: 3562 febrile patients were received. 995 (27.934) were confirmed malaria (monthly mean positive=82.917; negative=213.917). Disparity was observed between proportion of confirmed malaria cases and those treated. The proportion of negative cases treated for malaria increased from 15.566% in 2006 to 60.466% in 2009 to 12.500% in 2012. Over this period 33.043% of patients were over treated for malaria. The mean annual drug pressure was 169.857 (ACT), 135.287 (quinine) and 160.714 (antibiotics). Children age 1-4 and women 15-44 received the highest drug pressure. We calculated wasted resources (1,150,700USD) due to inappropriate treatment as a product of average cost of malaria treatment using ACT (2USD) and quinine (17USD) and annual drug pressures.

Discussion: Non-adherence to guidelines poses enormous household and government financial burden.

Implications for guideline developers/users: There is need to narrow the gap between guidelines developers and users in primary healthcare malaria case management.

ANNEXSURE 3

Interpretation of Results

Interpretation of Results

- **Test Proportions:**

Create proportions: $\left(\frac{\# \text{ Malaria+}}{\# \text{ Tested for Malaria}}\right)$ per year

Interpretation: Use the 95% CI for each proportion to determine whether the proportion is significant – CI that do not contain zero are indicative of proportions significantly different from zero.

- **Assess trend:**

Interpretation:

R^2 : indicates how well trend fits the data and $R^2 \times 100$ represents the % variance explained:

Regression p-value: indicates whether the model fits the better than simply taking the average. If the p-value ≤ 0.05 then the model is more appropriate than the average.

Parameters: Parameter values are used to construct a model

Linear: Proportion = B(constant) + B(year)xYear

Quadratic: Proportion = B(constant) + B(year)xYear+ B(year**2)xYearxYear

Cubic: Proportion = B(constant) + B(year)xYear+ B(year**2)xYearxYear +

B(year**3)xYearxYearxYear

Standardized Parameters: are used for quadratic and cubic trends to compare relative importance of model terms (e.g. Year vs. YearxYear vs. YearxYearxYear).

P-values for parameters: used to test whether parameters estimates (B-values) are significantly different from zero. If the p-value ≤ 0.05 then the associated term should remain in the model.

- **Compare Groups on number of Malaria positive cases**

Interpretation:

Significance of association: The Pearson Chi-Square (appropriate for small samples). The “Likelihood ratio” is used for data that are loglinear in nature. “Fisher’s Exact Test” is often used as an alternative to the Pearson X² when one or more cells contain less than 5 observations (Pearson Chi-Square results when one or more cells contain less than 5 observations cannot be trusted – at least 80% must be more than 5). The “Linear-by-Linear Association” statistic is used when the variables are ordinal.

If p-value (which ever selected is ≤ 0.05 then the association is statistically significant), else any relationship is mainly due to sampling error.

Phi or Cramer’s V Measure STRENGTH of ASSOCIATION between 2 categorical variables (practical significance). Phi: 2xn tables & Cramer’s V: nxn tables. Guidelines: $w=0.1$ =Small effect=No practically significant association | $w=0.3$ =Medium effect=practically visible association | $w=0.5$ =Large effect=practically significant association.

Compare column proportions: Each subscript letter in the crosstabs table denotes a subset of categories/ columns whose proportions **do not** differ significantly from each other at the 0.05 level. Test z-test for differences in proportions (adjusted for multiple comparisons using a Bonferroni adjustment).

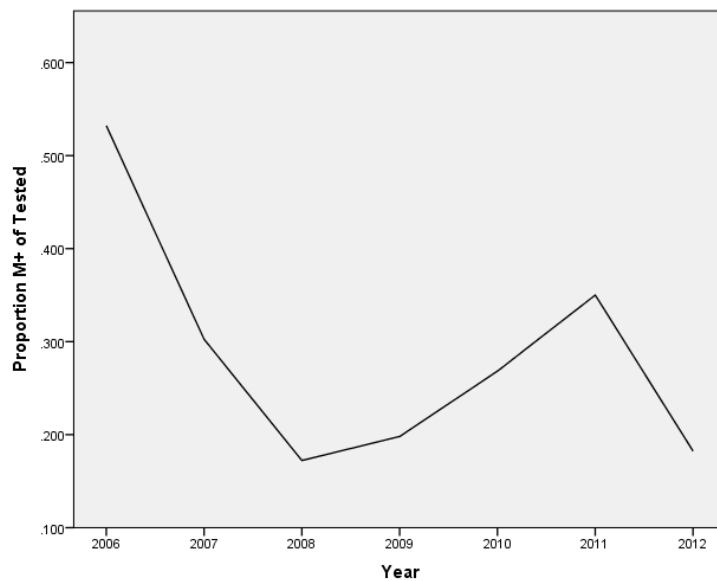
ANNEXSURE 4

Malaria Admissions Results

Malaria prevalence results

Years

Year	CNT_POS	CNT_TST	Proportion M+ of Tested	CI_Factor	CI95_Upper	CI95_Lower
2006	356	669	.532	.04	.57	.49
2007	187	618	.303	.04	.34	.27
2008	93	540	.172	.03	.20	.14
2009	103	520	.198	.03	.23	.16
2010	165	615	.268	.04	.30	.23
2011	244	697	.350	.04	.39	.31
2012	91	499	.182	.03	.22	.15



Tend	R-Squared	Regression Significance (p-value)	Parameter	Estimate	Standardized Parameter Estimate	Parameter Significance (p-value)
Linear	.272	.230	Linear	-.031	-.522	.230
			Constant/Intercept	61.868		.228
Quadratic	TOO FEW CASES		Linear			

Quadratic

Constant/Intercept

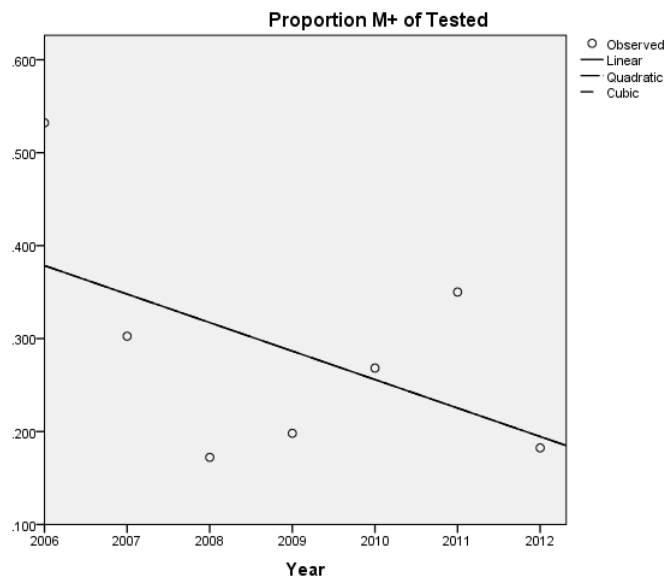
Linear

Quadratic

Cubic

Constant/Intercept

Cubic



Year vs. Age

Statistic	Value
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	.401
Phi	.355
Cramer's V	.159
Cells with expected count less than 5 (%)	21.4

Year vs. Gender

Statistic	Value
Pearson Chi-Square (p-value)	.042
Likelihood Ratio (p-value)	.039
Linear-by-Linear Association (p-value)	.337
Phi	.103
Cramer's V	.103
Cells with expected count less than 5 (%)	0

Months

Page 1

Month	CNT_POS	CNT_TST	Proportion M+ of Tested	CI_Factor	CI95_Upper	CI95_Lower
1	95	392	.242	.04	.28	.20
2	71	252	.282	.06	.34	.23
3	129	474	.272	.04	.31	.23
4	104	335	.310	.05	.36	.26
5	114	358	.318	.05	.37	.27
6	170	442	.385	.05	.43	.34
7	133	455	.292	.04	.33	.25
8	101	284	.356	.06	.41	.30
9	71	300	.237	.05	.28	.19
10	90	323	.279	.05	.33	.23
11	62	231	.268	.06	.33	.21
12	99	312	.317	.05	.37	.27

Months vs. Age

Statistic	Value
Pearson Chi-Square (p-value)	.105
Likelihood Ratio (p-value)	.070
Linear-by-Linear Association (p-value)	.902
Phi	.235
Cramer's V	.105
Cells with expected count less than 5 (%)	30.6

Months vs. Gender

Statistic	Value
Pearson Chi-Square (p-value)	.904
Likelihood Ratio (p-value)	.902
Linear-by-Linear Association (p-value)	.524
Phi	.067
Cramer's V	.067
Cells with expected count less than 5 (%)	0

Months x Years

Page

1

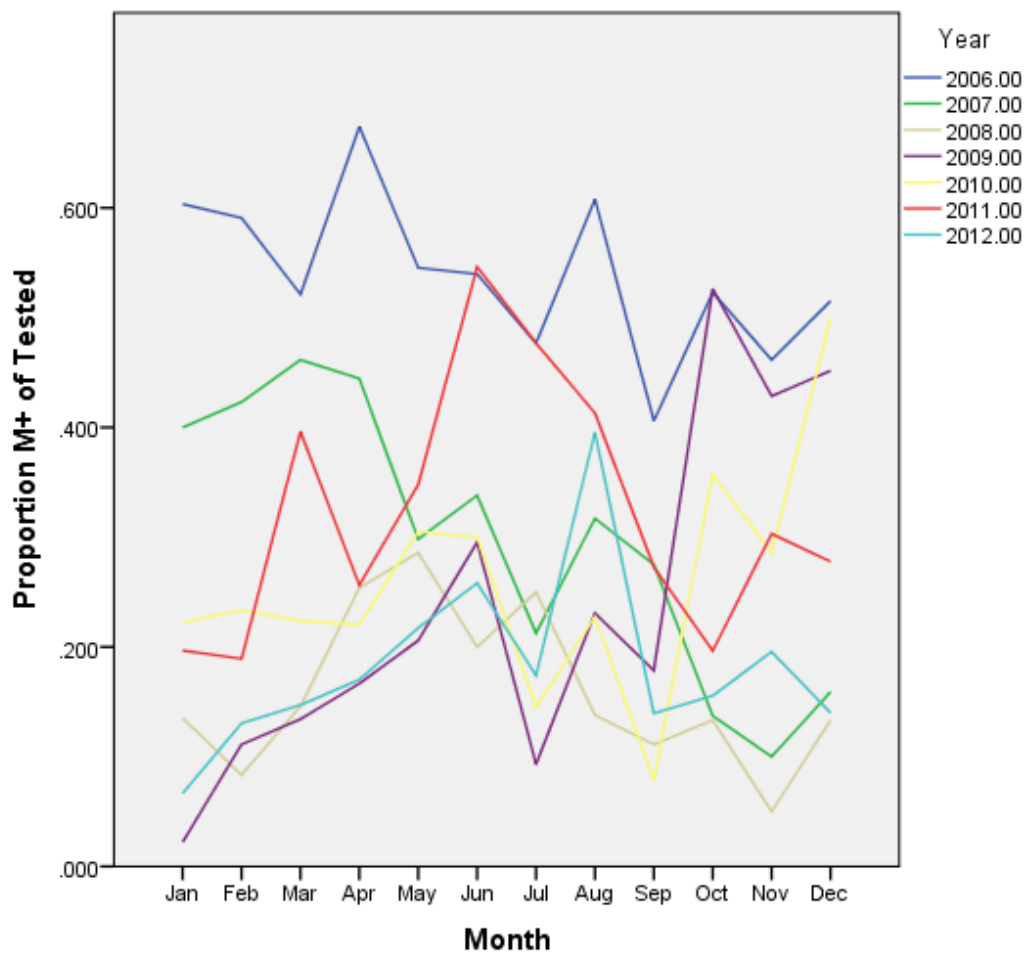
Year	Month	CNT_POS	CNT_TST	Proportion M+ of Tested	CI_Factor	CI95_Upper	CI95_Lower
2006	1	35	58	.603	.13	.73	.48
2006	2	26	44	.591	.15	.74	.45
2006	3	37	71	.521	.12	.64	.40
2006	4	31	46	.674	.14	.81	.54
2006	5	24	44	.545	.15	.69	.40
2006	6	34	63	.540	.12	.66	.42
2006	7	41	86	.477	.11	.58	.37
2006	8	31	51	.608	.13	.74	.47
2006	9	28	69	.406	.12	.52	.29
2006	10	34	65	.523	.12	.64	.40
2006	11	18	39	.462	.16	.62	.31
2006	12	17	33	.515	.17	.69	.34
2007	1	22	55	.400	.13	.53	.27
2007	2	22	52	.423	.13	.56	.29
2007	3	24	52	.462	.14	.60	.33
2007	4	20	45	.444	.15	.59	.30
2007	5	17	57	.298	.12	.42	.18
2007	6	24	71	.338	.11	.45	.23
2007	7	17	80	.213	.09	.30	.12
2007	8	13	41	.317	.14	.46	.17
2007	9	11	40	.275	.14	.41	.14
2007	10	7	51	.137	.09	.23	.04
2007	11	3	30	.100	.11	.21	-.01
2007	12	7	44	.159	.11	.27	.05
2008	1	10	74	.135	.08	.21	.06
2008	2	4	48	.083	.08	.16	.01
2008	3	7	48	.146	.10	.25	.05

2008	4	16	63	.254	.11	.36	.15
2008	5	14	49	.286	.13	.41	.16
2008	6	11	55	.200	.11	.31	.09
2008	7	13	52	.250	.12	.37	.13
2008	8	4	29	.138	.13	.26	.01

Year	Month	CNT_POS	CNT_TST	Proportion M+ of Tested	CI_Factor	CI95_Upper	CI95_Lower
2008	9	3	27	.111	.12	.23	-.01
2008	10	6	45	.133	.10	.23	.03
2008	11	1	20	.050	.10	.15	-.05
2008	12	4	30	.133	.12	.25	.01
2009	1	1	45	.022	.04	.07	-.02
2009	2	2	18	.111	.15	.26	-.03
2009	3	20	149	.134	.05	.19	.08
2009	4	6	36	.167	.12	.29	.04
2009	5	7	34	.206	.14	.34	.07
2009	6	13	44	.295	.13	.43	.16
2009	7	4	43	.093	.09	.18	.01
2009	8	12	52	.231	.11	.35	.12
2009	9	5	28	.179	.14	.32	.04
2009	10	10	19	.526	.22	.75	.30
2009	11	9	21	.429	.21	.64	.22
2009	12	14	31	.452	.18	.63	.28
2010	1	12	54	.222	.11	.33	.11
2010	2	7	30	.233	.15	.38	.08
2010	3	15	67	.224	.10	.32	.12
2010	4	13	59	.220	.11	.33	.11
2010	5	18	59	.305	.12	.42	.19
2010	6	21	70	.300	.11	.41	.19
2010	7	9	62	.145	.09	.23	.06
2010	8	5	22	.227	.18	.40	.05
2010	9	3	38	.079	.09	.16	-.01
2010	10	15	42	.357	.14	.50	.21
2010	11	12	42	.286	.14	.42	.15
2010	12	35	70	.500	.12	.62	.38
2011	1	12	61	.197	.10	.30	.10
2011	2	7	37	.189	.13	.32	.06
2011	3	21	53	.396	.13	.53	.26
2011	4	10	39	.256	.14	.39	.12

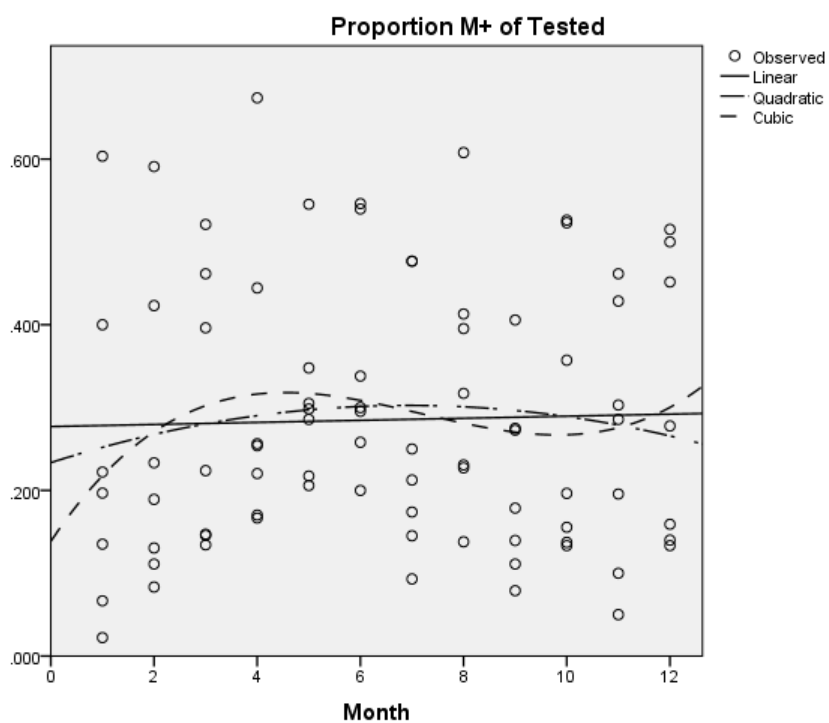
Year	Month	CNT_POS	CNT_TST	Proportion M+ of Tested	CI_Factor	CI95_Upper	CI95_Lower
2011	5	24	69	.348	.11	.46	.24
2011	6	59	108	.546	.09	.64	.45
2011	7	41	86	.477	.11	.58	.37
2011	8	19	46	.413	.14	.56	.27

2011	9	15	55	.273	.12	.39	.16
2011	10	11	56	.196	.10	.30	.09
2011	11	10	33	.303	.16	.46	.15
2011	12	15	54	.278	.12	.40	.16
2012	1	3	45	.067	.07	.14	-.01
2012	2	3	23	.130	.14	.27	-.01
2012	3	5	34	.147	.12	.27	.03
2012	4	8	47	.170	.11	.28	.06
2012	5	10	46	.217	.12	.34	.10
2012	6	8	31	.258	.15	.41	.10
2012	7	8	46	.174	.11	.28	.06
2012	8	17	43	.395	.15	.54	.25
2012	9	6	43	.140	.10	.24	.04
2012	10	7	45	.156	.11	.26	.05
2012	11	9	46	.196	.11	.31	.08
2012	12	7	50	.140	.10	.24	.04

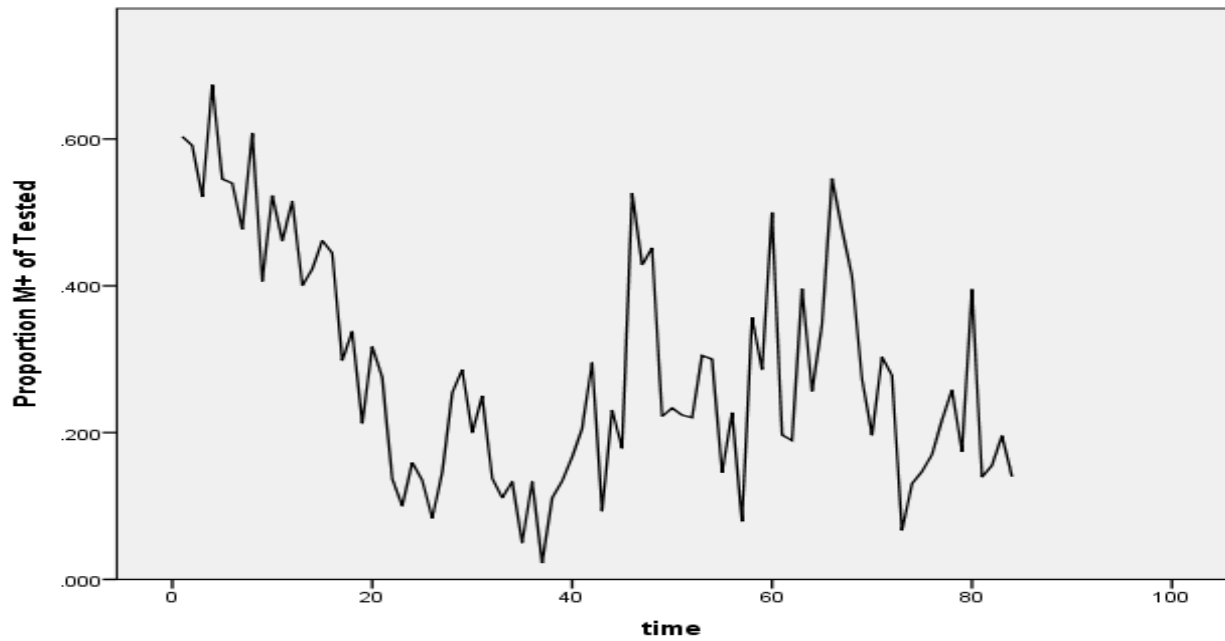


Tend	R-Squared	Regression Significance (p-value)	Parameter	Estimate	Standardized Parameter Estimate	Parameter Significance (p-value)
Linear	.001	.803	Linear	.001	.028	.803

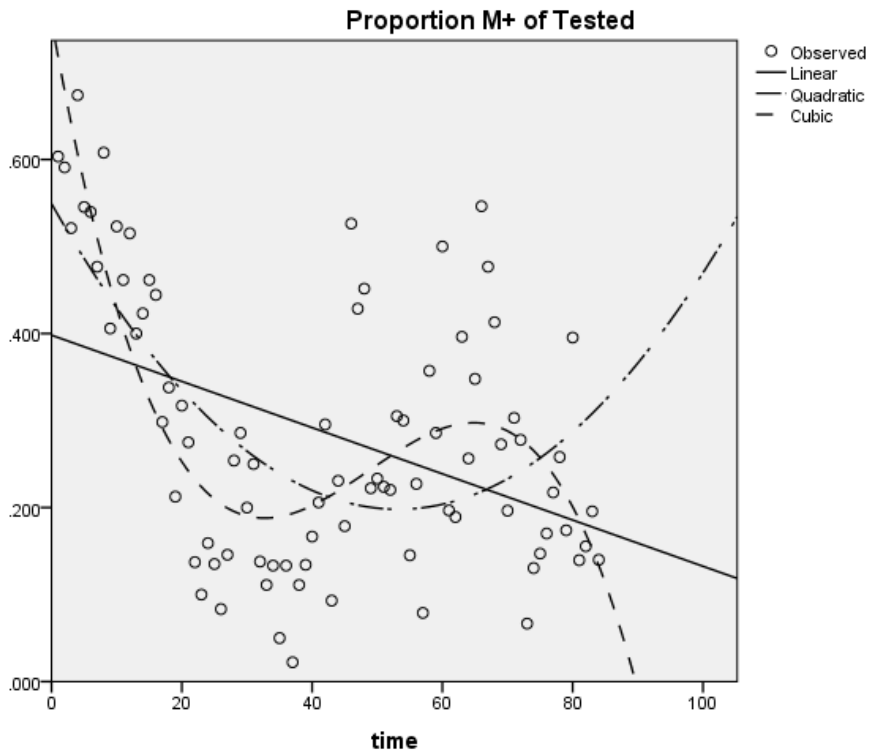
			Constant/Intercept	.277		<0.001
			Linear	.020	.441	.365
Quadratic	.010	.661	Quadratic	-.001	-.424	.383
			Constant/Intercept	.234		<0.001
			Linear	.093	2.067	.138
Cubic	.029	.493	Quadratic	-.015	-4.438	.172
			Cubic	.001	2.463	.212
			Constant/Intercept	.139		.159



Trend over time



Tend	R-Squared	Regression Significance (p-value)	Parameter	Estimate	Standardized Parameter Estimate	Parameter Significance (p-value)
Linear	.170	.000	Linear	-.003	-.413	<0.001
			Constant/Intercept	.398		<0.001
Quadratic	.345	.000	Linear	-.013	-2.052	<0.001
			Quadratic	.000	1.692	<0.001
			Constant/Intercept	.549		<0.001
Cubic	.571	.000	Linear	-.042	-6.536	<0.001
			Quadratic	.001	13.192	<0.001
			Cubic	-6.613E-06	-7.256	<0.001
			Constant/Intercept	.759		<0.001



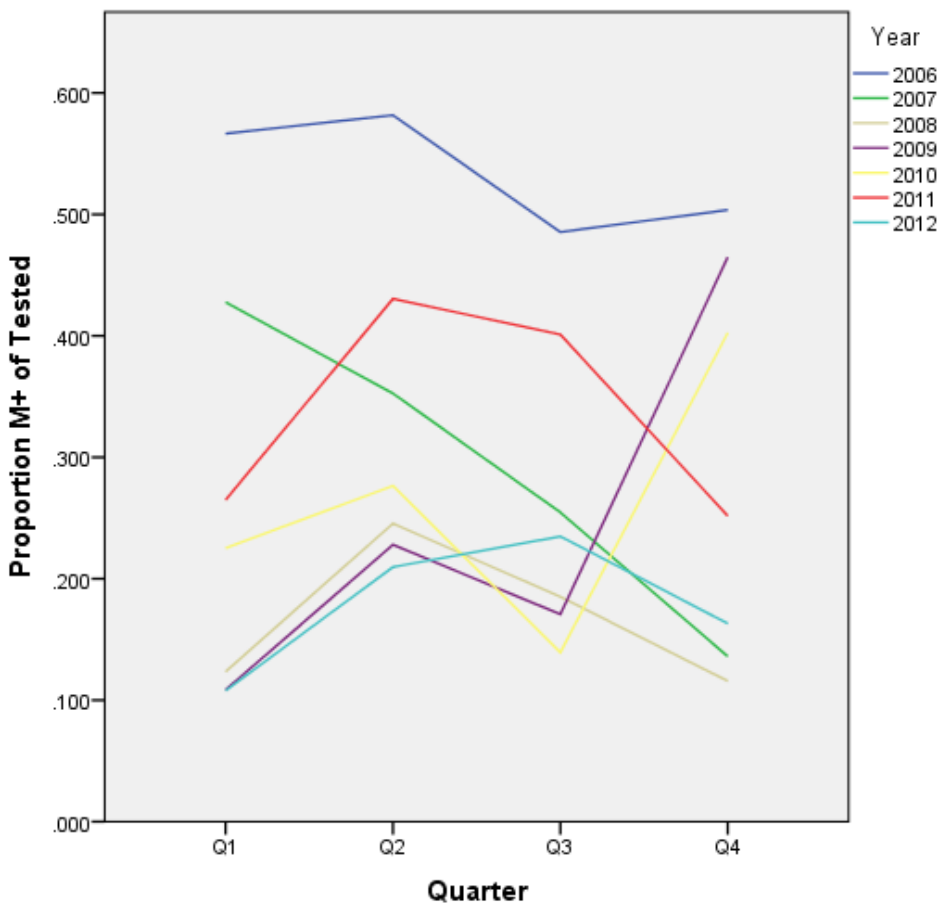
Quarters

Q	CNT_POS	CNT_TST	proppos	CI_Factor	CI95_Upper	CI95_Lower
1	295	1118	.264	.03	.29	.24
2	388	1135	.342	.03	.37	.31
3	305	1039	.294	.03	.32	.27
4	251	866	.290	.03	.32	.26

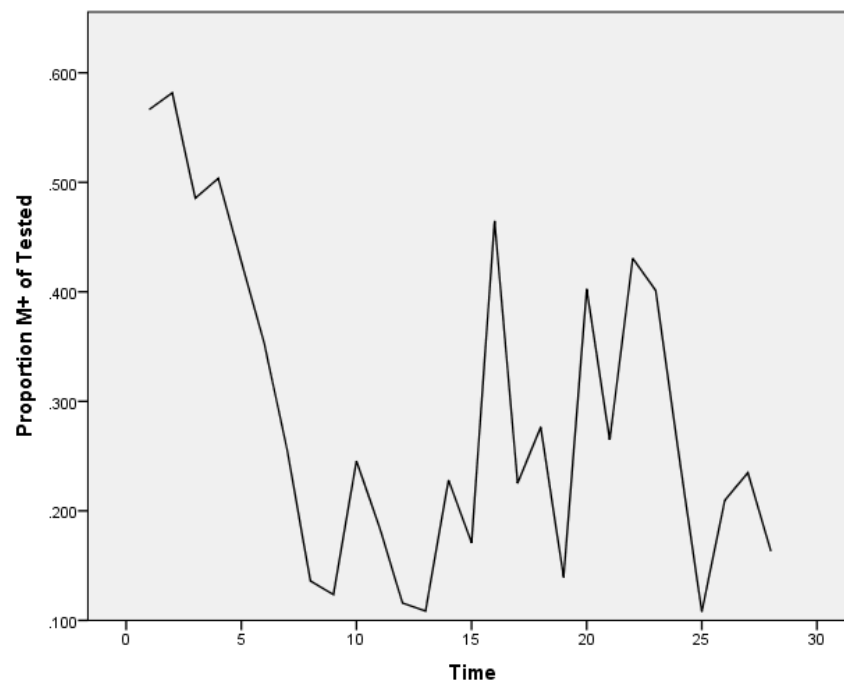
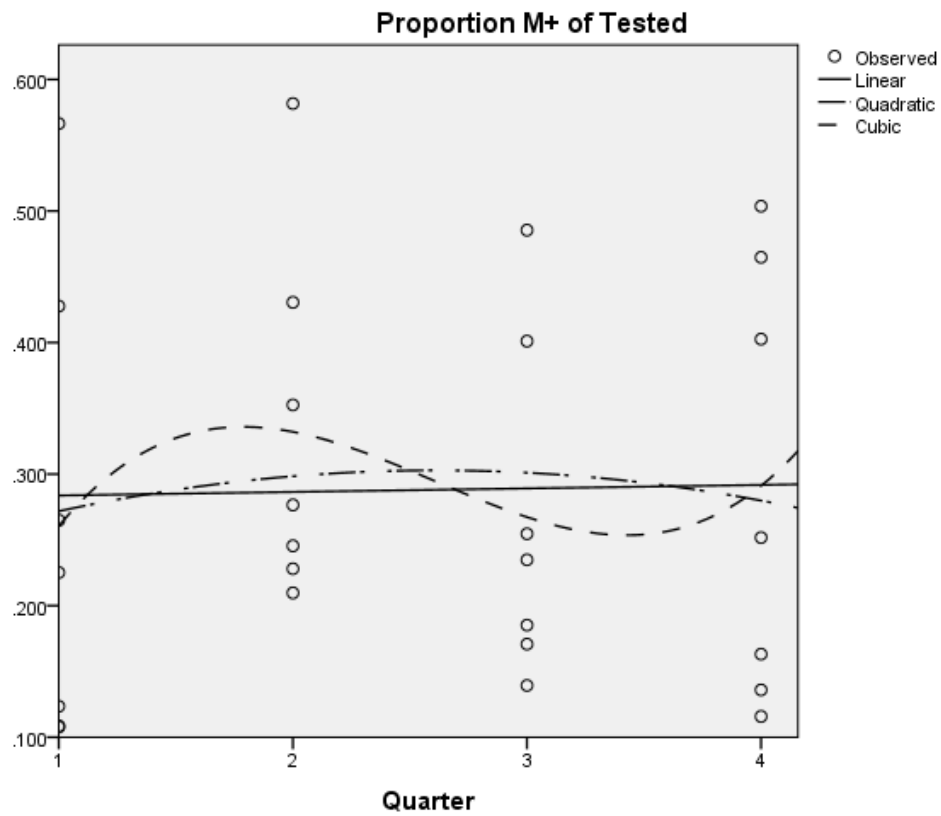
Quarters X Years

Year	Quarter	Count M+	CNT_TST	Proportion M+ of Tested	CI_Factor	CI95_Upper	CI95_Lower
2006	1	98	173	.566	.07	.64	.49
2006	2	89	153	.582	.08	.66	.50
2006	3	100	206	.485	.07	.55	.42
2006	4	69	137	.504	.08	.59	.42
2007	1	68	159	.428	.08	.50	.35
2007	2	61	173	.353	.07	.42	.28
2007	3	41	161	.255	.07	.32	.19
2007	4	17	125	.136	.06	.20	.08

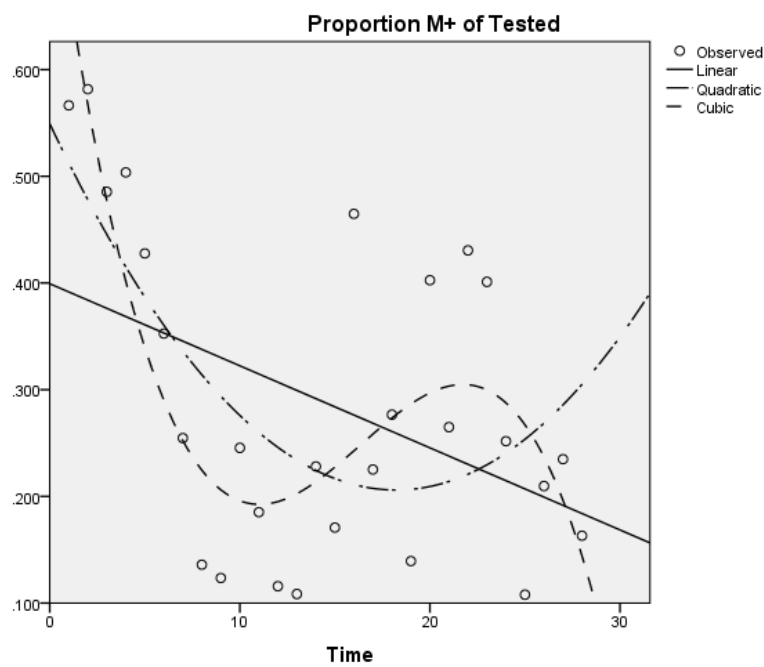
2008	1	21	170	.124	.05	.17	.07
2008	2	41	167	.246	.07	.31	.18
2008	3	20	108	.185	.07	.26	.11
2008	4	11	95	.116	.06	.18	.05
2009	1	23	212	.108	.04	.15	.07
2009	2	26	114	.228	.08	.31	.15
2009	3	21	123	.171	.07	.24	.10
2009	4	33	71	.465	.12	.58	.35
2010	1	34	151	.225	.07	.29	.16
2010	2	52	188	.277	.06	.34	.21
2010	3	17	122	.139	.06	.20	.08
2010	4	62	154	.403	.08	.48	.33
2011	1	40	151	.265	.07	.34	.19
2011	2	93	216	.431	.07	.50	.36
2011	3	75	187	.401	.07	.47	.33
2011	4	36	143	.252	.07	.32	.18
2012	1	11	102	.108	.06	.17	.05
2012	2	26	124	.210	.07	.28	.14
2012	3	31	132	.235	.07	.31	.16
2012	4	23	141	.163	.06	.22	.10



Tend	R-Squared	Regression Significance (p-value)	Parameter	Estimate	Standardized Parameter Estimate	Parameter Significance (p-value)
Linear	.000	.916	Linear	.003	.021	.916
			Constant/Intercept	.281		<0.001
Quadratic	.007	.912	Linear	.062	.485	.672
			Quadratic	-.012	-.472	.680
			Constant/Intercept	.221		.177
Cubic	.038	.814	Linear	.688	5.353	.356
			Quadratic	-.293	-11.577	.373
			Cubic	.037	6.374	.391
			Constant/Intercept	-.172		.722



Tend	R-Squared	Regression Significance (p-value)	Parameter	Estimate	Standardized Parameter Estimate	Parameter Significance (p-value)
Linear	.187	.022	Linear	-.008	-.432	.022
			Constant/Intercept	.399		<0.001
Quadratic	.363	.004	Linear	-.038	-2.118	.004
			Quadratic	.001	1.737	.015
			Constant/Intercept	.549		<0.001
Cubic	.638	.000	Linear	-.132	-7.408	<0.001
			Quadratic	.009	15.127	<0.001
			Cubic	.000	-8.371	<0.001
			Constant/Intercept	.796		<0.001



Year x Month Trend (...within age groups)

AgeCat	Year	CNT_POS	CNT_TST	proppos	time	CI_Factor	CI95_Upper	CI95_Lower
1	2006	7	13	.54	1	.27	.81	.27
1	2006	3	5	.60	2	.43	1.03	.17
1	2006	6	12	.50	3	.28	.78	.22
1	2006	5	7	.71	4	.33	1.05	.38
1	2006	1	1	1.00	5	.00	1.00	1.00
1	2006	2	5	.40	6	.43	.83	-.03
1	2006	5	10	.50	7	.31	.81	.19
1	2006	3	6	.50	8	.40	.90	.10
1	2006	0	5	.00	9	.00	.00	.00
1	2006	7	10	.70	10	.28	.98	.42
1	2006	4	8	.50	11	.35	.85	.15
1	2006	2	5	.40	12	.43	.83	-.03
1	2007	4	8	.50	13	.35	.85	.15
1	2007	2	3	.67	14	.53	1.20	.13
1	2007	0	1	.00	15	.00	.00	.00
1	2007	1	3	.33	16	.53	.87	-.20
1	2007	0	3	.00	17	.00	.00	.00
1	2007	0	5	.00	18	.00	.00	.00
1	2007	0	6	.00	19	.00	.00	.00
1	2007	1	5	.20	20	.35	.55	-.15
1	2007	1	5	.20	21	.35	.55	-.15
1	2007	0	6	.00	22	.00	.00	.00
1	2007	0	4	.00	23	.00	.00	.00
1	2007	0	6	.00	24	.00	.00	.00
1	2008	1	9	.11	25	.21	.32	-.09
1	2008	0	9	.00	26	.00	.00	.00
1	2008	0	2	.00	27	.00	.00	.00
1	2008	1	9	.11	28	.21	.32	-.09
1	2008	1	6	.17	29	.30	.46	-.13
1	2008	1	1	1.00	30	.00	1.00	1.00
1	2008	0	3	.00	31	.00	.00	.00
1	2008	0	2	.00	32	.00	.00	.00
1	2008	0	6	.00	33	.00	.00	.00
1	2008	1	11	.09	34	.17	.26	-.08
1	2008	0	4	.00	35	.00	.00	.00
1	2008	0	3	.00	36	.00	.00	.00
1	2009	0	6	.00	37	.00	.00	.00
1	2009	0	4	.00	38	.00	.00	.00
1	2009	0	24	.00	39	.00	.00	.00
1	2009	0	4	.00	40	.00	.00	.00
1	2009	0	8	.00	41	.00	.00	.00
1	2009	0	5	.00	42	.00	.00	.00
1	2009	0	6	.00	43	.00	.00	.00

1	2009	0	2	.00	44	.00	.00	.00
1	2009		0		45			
1	2009	2	2	1.00	46	.00	1.00	1.00
1	2009	1	1	1.00	47	.00	1.00	1.00
1	2010	0	4	.00	49	.00	.00	.00
1	2010	0	5	.00	50	.00	.00	.00
1	2010	0	8	.00	51	.00	.00	.00
1	2010	1	9	.11	52	.21	.32	-.09
1	2010	1	3	.33	53	.53	.87	-.20
1	2010	3	5	.60	54	.43	1.03	.17
1	2010	1	4	.25	55	.42	.67	-.17
1	2010	0	1	.00	56	.00	.00	.00
1	2010	0	4	.00	57	.00	.00	.00
1	2010	0	3	.00	58	.00	.00	.00
1	2010	0	1	.00	59	.00	.00	.00
1	2010	1	2	.50	60	.69	1.19	-.19
1	2011	1	7	.14	61	.26	.40	-.12
1	2011	0	1	.00	62	.00	.00	.00
1	2011	0	2	.00	63	.00	.00	.00
1	2011	0	2	.00	64	.00	.00	.00
1	2011	1	1	1.00	65	.00	1.00	1.00
1	2011	0	1	.00	66	.00	.00	.00
1	2011	1	3	.33	67	.53	.87	-.20
1	2011	0	3	.00	68	.00	.00	.00
1	2011	0	6	.00	69	.00	.00	.00
1	2011	0	2	.00	70	.00	.00	.00
1	2011	0	3	.00	71	.00	.00	.00
1	2011	3	7	.43	72	.37	.80	.06
1	2012	0	4	.00	73	.00	.00	.00
1	2012	0	2	.00	74	.00	.00	.00
1	2012	0	6	.00	75	.00	.00	.00
1	2012	0	2	.00	76	.00	.00	.00
1	2012	0	2	.00	77	.00	.00	.00
1	2012	1	3	.33	78	.53	.87	-.20
1	2012	0	7	.00	79	.00	.00	.00
1	2012	0	3	.00	80	.00	.00	.00
1	2012	0	2	.00	81	.00	.00	.00
1	2012	0	2	.00	82	.00	.00	.00
1	2012	1	4	.25	83	.42	.67	-.17
1	2012	0	7	.00	84	.00	.00	.00
2	2006	11	16	.69	1	.23	.91	.46
2	2006	5	9	.56	2	.32	.88	.23
2	2006	12	20	.60	3	.21	.81	.39
2	2006	10	15	.67	4	.24	.91	.43

2	2006	9	12	.75	5	.25	1.00	.51
2	2006	11	18	.61	6	.23	.84	.39
2	2006	13	25	.52	7	.20	.72	.32
2	2006	17	19	.89	8	.14	1.03	.76
2	2006	11	16	.69	9	.23	.91	.46
2	2006	9	13	.69	10	.25	.94	.44
2	2006	8	10	.80	11	.25	1.05	.55
2	2006	8	9	.89	12	.21	1.09	.68
2	2007	8	13	.62	13	.26	.88	.35
2	2007	7	13	.54	14	.27	.81	.27
2	2007	4	6	.67	15	.38	1.04	.29
2	2007	9	12	.75	16	.25	1.00	.51
2	2007	9	26	.35	17	.18	.53	.16
2	2007	9	22	.41	18	.21	.61	.20
2	2007	1	22	.05	19	.09	.13	-.04
2	2007	1	8	.13	20	.23	.35	-.10
2	2007	4	9	.44	21	.32	.77	.12
2	2007	3	15	.20	22	.20	.40	.00
2	2007	1	10	.10	23	.19	.29	-.09
2	2007	1	13	.08	24	.14	.22	-.07
2	2008	2	18	.11	25	.15	.26	-.03
2	2008	1	9	.11	26	.21	.32	-.09
2	2008	4	16	.25	27	.21	.46	.04
2	2008	2	13	.15	28	.20	.35	-.04
2	2008	0	12	.00	29	.00	.00	.00
2	2008	2	14	.14	30	.18	.33	-.04
2	2008	2	6	.33	31	.38	.71	-.04
2	2008	0	5	.00	32	.00	.00	.00
2	2008	0	5	.00	33	.00	.00	.00
2	2008	1	11	.09	34	.17	.26	-.08
2	2008	0	2	.00	35	.00	.00	.00
2	2008	1	9	.11	36	.21	.32	-.09
2	2009	0	8	.00	37	.00	.00	.00
2	2009	0	1	.00	38	.00	.00	.00
2	2009	2	20	.10	39	.13	.23	-.03
2	2009	2	10	.20	40	.25	.45	-.05
2	2009	2	5	.40	41	.43	.83	-.03
2	2009	3	7	.43	42	.37	.80	.06
2	2009	2	10	.20	43	.25	.45	-.05
2	2009	5	13	.38	44	.26	.65	.12
2	2009	0	8	.00	45	.00	.00	.00
2	2009	5	8	.63	46	.34	.96	.29
2	2009	5	11	.45	47	.29	.75	.16
2	2009	2	7	.29	48	.33	.62	-.05

2	2010	1	8	.13	49	.23	.35	-.10
2	2010	1	6	.17	50	.30	.46	-.13
2	2010	6	19	.32	51	.21	.52	.11
2	2010	8	17	.47	52	.24	.71	.23
2	2010	5	18	.28	53	.21	.48	.07
2	2010	7	18	.39	54	.23	.61	.16
2	2010	2	11	.18	55	.23	.41	-.05
2	2010	1	3	.33	56	.53	.87	-.20
2	2010	1	5	.20	57	.35	.55	-.15
2	2010	3	7	.43	58	.37	.80	.06
2	2010	2	4	.50	59	.49	.99	.01
2	2010	6	15	.40	60	.25	.65	.15
2	2011	4	11	.36	61	.28	.65	.08
2	2011	0	4	.00	62	.00	.00	.00
2	2011	7	14	.50	63	.26	.76	.24
2	2011	5	12	.42	64	.28	.70	.14
2	2011	8	17	.47	65	.24	.71	.23
2	2011	19	27	.70	66	.17	.88	.53
2	2011	14	28	.50	67	.19	.69	.31
2	2011	6	8	.75	68	.30	1.05	.45
2	2011	6	16	.38	69	.24	.61	.14
2	2011	4	13	.31	70	.25	.56	.06
2	2011	4	6	.67	71	.38	1.04	.29
2	2011	7	18	.39	72	.23	.61	.16
2	2012	3	10	.30	73	.28	.58	.02
2	2012	0	4	.00	74	.00	.00	.00
2	2012	1	7	.14	75	.26	.40	-.12
2	2012	2	10	.20	76	.25	.45	-.05
2	2012	5	11	.45	77	.29	.75	.16
2	2012	2	3	.67	78	.53	1.20	.13
2	2012	0	6	.00	79	.00	.00	.00
2	2012	2	6	.33	80	.38	.71	-.04
2	2012	2	7	.29	81	.33	.62	-.05
2	2012	2	9	.22	82	.27	.49	-.05
2	2012	2	10	.20	83	.25	.45	-.05
2	2012	1	8	.13	84	.23	.35	-.10
3	2006	4	6	.67	1	.38	1.04	.29
3	2006	4	7	.57	2	.37	.94	.20
3	2006	2	3	.67	3	.53	1.20	.13
3	2006	5	5	1.00	4	.00	1.00	1.00
3	2006	4	7	.57	5	.37	.94	.20
3	2006	7	10	.70	6	.28	.98	.42
3	2006	2	11	.18	7	.23	.41	-.05
3	2006	1	4	.25	8	.42	.67	-.17

3	2006	4	12	.33	9	.27	.60	.07
3	2006	3	5	.60	10	.43	1.03	.17
3	2006	2	4	.50	11	.49	.99	.01
3	2006	1	2	.50	12	.69	1.19	-.19
3	2007	4	6	.67	13	.38	1.04	.29
3	2007	4	8	.50	14	.35	.85	.15
3	2007	4	9	.44	15	.32	.77	.12
3	2007	4	11	.36	16	.28	.65	.08
3	2007	3	10	.30	17	.28	.58	.02
3	2007	9	15	.60	18	.25	.85	.35
3	2007	5	11	.45	19	.29	.75	.16
3	2007	3	3	1.00	20	.00	1.00	1.00
3	2007	1	3	.33	21	.53	.87	-.20
3	2007	1	5	.20	22	.35	.55	-.15
3	2007	2	7	.29	23	.33	.62	-.05
3	2007	1	5	.20	24	.35	.55	-.15
3	2008	2	15	.13	25	.17	.31	-.04
3	2008	2	5	.40	26	.43	.83	-.03
3	2008	1	10	.10	27	.19	.29	-.09
3	2008	5	12	.42	28	.28	.70	.14
3	2008	4	12	.33	29	.27	.60	.07
3	2008	4	17	.24	30	.20	.44	.03
3	2008	4	8	.50	31	.35	.85	.15
3	2008	1	4	.25	32	.42	.67	-.17
3	2008	1	3	.33	33	.53	.87	-.20
3	2008	0	3	.00	34	.00	.00	.00
3	2008	1	2	.50	35	.69	1.19	-.19
3	2008	3	6	.50	36	.40	.90	.10
3	2009	0	7	.00	37	.00	.00	.00
3	2009	1	3	.33	38	.53	.87	-.20
3	2009	6	28	.21	39	.15	.37	.06
3	2009	0	1	.00	40	.00	.00	.00
3	2009	0	5	.00	41	.00	.00	.00
3	2009	5	13	.38	42	.26	.65	.12
3	2009	2	6	.33	43	.38	.71	-.04
3	2009	3	10	.30	44	.28	.58	.02
3	2009	1	3	.33	45	.53	.87	-.20
3	2009	0	4	.00	46	.00	.00	.00
3	2009	0	2	.00	47	.00	.00	.00
3	2009	2	5	.40	48	.43	.83	-.03
3	2010	0	3	.00	49	.00	.00	.00
3	2010	0	3	.00	50	.00	.00	.00
3	2010	3	11	.27	51	.26	.54	.01
3	2010	0	7	.00	52	.00	.00	.00

3	2010	3	15	.20	53	.20	.40	.00
3	2010	5	18	.28	54	.21	.48	.07
3	2010	3	16	.19	55	.19	.38	.00
3	2010	1	4	.25	56	.42	.67	-.17
3	2010	1	4	.25	57	.42	.67	-.17
3	2010	3	6	.50	58	.40	.90	.10
3	2010	5	13	.38	59	.26	.65	.12
3	2010	12	17	.71	60	.22	.92	.49
3	2011	4	14	.29	61	.24	.52	.05
3	2011	4	12	.33	62	.27	.60	.07
3	2011	8	15	.53	63	.25	.79	.28
3	2011	4	6	.67	64	.38	1.04	.29
3	2011	9	22	.41	65	.21	.61	.20
3	2011	27	38	.71	66	.14	.85	.57
3	2011	19	26	.73	67	.17	.90	.56
3	2011	10	12	.83	68	.21	1.04	.62
3	2011	7	13	.54	69	.27	.81	.27
3	2011	4	16	.25	70	.21	.46	.04
3	2011	3	9	.33	71	.31	.64	.03
3	2011	4	6	.67	72	.38	1.04	.29
3	2012	0	4	.00	73	.00	.00	.00
3	2012	2	3	.67	74	.53	1.20	.13
3	2012	2	5	.40	75	.43	.83	-.03
3	2012	2	11	.18	76	.23	.41	-.05
3	2012	2	8	.25	77	.30	.55	-.05
3	2012	0	5	.00	78	.00	.00	.00
3	2012	4	7	.57	79	.37	.94	.20
3	2012	8	10	.80	80	.25	1.05	.55
3	2012	3	7	.43	81	.37	.80	.06
3	2012	2	9	.22	82	.27	.49	-.05
3	2012	3	6	.50	83	.40	.90	.10
3	2012	1	6	.17	84	.30	.46	-.13
4	2006	10	18	.56	1	.23	.79	.33
4	2006	11	19	.58	2	.22	.80	.36
4	2006	15	28	.54	3	.18	.72	.35
4	2006	9	14	.64	4	.25	.89	.39
4	2006	9	21	.43	5	.21	.64	.22
4	2006	8	20	.40	6	.21	.61	.19
4	2006	17	33	.52	7	.17	.69	.34
4	2006	9	19	.47	8	.22	.70	.25
4	2006	11	30	.37	9	.17	.54	.19
4	2006	12	30	.40	10	.18	.58	.22
4	2006	4	16	.25	11	.21	.46	.04
4	2006	6	16	.38	12	.24	.61	.14

4	2007	5	17	.29	13	.22	.51	.08
4	2007	8	19	.42	14	.22	.64	.20
4	2007	11	26	.42	15	.19	.61	.23
4	2007	5	17	.29	16	.22	.51	.08
4	2007	4	15	.27	17	.22	.49	.04
4	2007	4	24	.17	18	.15	.32	.02
4	2007	6	28	.21	19	.15	.37	.06
4	2007	7	23	.30	20	.19	.49	.12
4	2007	5	20	.25	21	.19	.44	.06
4	2007	3	23	.13	22	.14	.27	-.01
4	2007	0	6	.00	23	.00	.00	.00
4	2007	3	18	.17	24	.17	.34	-.01
4	2008	5	27	.19	25	.15	.33	.04
4	2008	1	22	.05	26	.09	.13	-.04
4	2008	2	19	.11	27	.14	.24	-.03
4	2008	8	27	.30	28	.17	.47	.12
4	2008	8	17	.47	29	.24	.71	.23
4	2008	4	20	.20	30	.18	.38	.02
4	2008	7	32	.22	31	.14	.36	.08
4	2008	3	15	.20	32	.20	.40	.00
4	2008	1	10	.10	33	.19	.29	-.09
4	2008	3	14	.21	34	.21	.43	.00
4	2008	0	11	.00	35	.00	.00	.00
4	2008	0	10	.00	36	.00	.00	.00
4	2009	1	22	.05	37	.09	.13	-.04
4	2009	1	9	.11	38	.21	.32	-.09
4	2009	10	62	.16	39	.09	.25	.07
4	2009	3	18	.17	40	.17	.34	-.01
4	2009	4	14	.29	41	.24	.52	.05
4	2009	5	17	.29	42	.22	.51	.08
4	2009	0	16	.00	43	.00	.00	.00
4	2009	3	22	.14	44	.14	.28	-.01
4	2009	4	13	.31	45	.25	.56	.06
4	2009	3	4	.75	46	.42	1.17	.33
4	2009	3	5	.60	47	.43	1.03	.17
4	2009	8	15	.53	48	.25	.79	.28
4	2010	10	31	.32	49	.16	.49	.16
4	2010	5	13	.38	50	.26	.65	.12
4	2010	6	25	.24	51	.17	.41	.07
4	2010	4	21	.19	52	.17	.36	.02
4	2010	7	20	.35	53	.21	.56	.14
4	2010	5	24	.21	54	.16	.37	.05
4	2010	1	24	.04	55	.08	.12	-.04
4	2010	1	9	.11	56	.21	.32	-.09

4	2010	1	19	.05	57	.10	.15	-.05
4	2010	7	19	.37	58	.22	.59	.15
4	2010	3	18	.17	59	.17	.34	-.01
4	2010	13	26	.50	60	.19	.69	.31
4	2011	3	20	.15	61	.16	.31	-.01
4	2011	3	18	.17	62	.17	.34	-.01
4	2011	6	17	.35	63	.23	.58	.13
4	2011	1	11	.09	64	.17	.26	-.08
4	2011	6	23	.26	65	.18	.44	.08
4	2011	9	28	.32	66	.17	.49	.15
4	2011	7	25	.28	67	.18	.46	.10
4	2011	2	16	.13	68	.16	.29	-.04
4	2011	1	15	.07	69	.13	.19	-.06
4	2011	2	14	.14	70	.18	.33	-.04
4	2011	3	12	.25	71	.25	.50	.01
4	2011	1	18	.06	72	.11	.16	-.05
4	2012	0	22	.00	73	.00	.00	.00
4	2012	1	14	.07	74	.13	.21	-.06
4	2012	2	15	.13	75	.17	.31	-.04
4	2012	4	20	.20	76	.18	.38	.02
4	2012	3	20	.15	77	.16	.31	-.01
4	2012	4	16	.25	78	.21	.46	.04
4	2012	4	19	.21	79	.18	.39	.03
4	2012	7	23	.30	80	.19	.49	.12
4	2012	1	20	.05	81	.10	.15	-.05
4	2012	2	20	.10	82	.13	.23	-.03
4	2012	3	14	.21	83	.21	.43	.00
4	2012	4	19	.21	84	.18	.39	.03
5	2006	3	5	.60	1	.43	1.03	.17
5	2006	2	3	.67	2	.53	1.20	.13
5	2006	2	8	.25	3	.30	.55	-.05
5	2006	2	5	.40	4	.43	.83	-.03
5	2006	1	2	.50	5	.69	1.19	-.19
5	2006	4	8	.50	6	.35	.85	.15
5	2006	3	6	.50	7	.40	.90	.10
5	2006	1	3	.33	8	.53	.87	-.20
5	2006	2	5	.40	9	.43	.83	-.03
5	2006	2	4	.50	10	.49	.99	.01
5	2006		0		11			
5	2006	0	1	.00	12	.00	.00	.00
5	2007	1	6	.17	13	.30	.46	-.13
5	2007	1	7	.14	14	.26	.40	-.12
5	2007	2	5	.40	15	.43	.83	-.03
5	2007	0	1	.00	16	.00	.00	.00

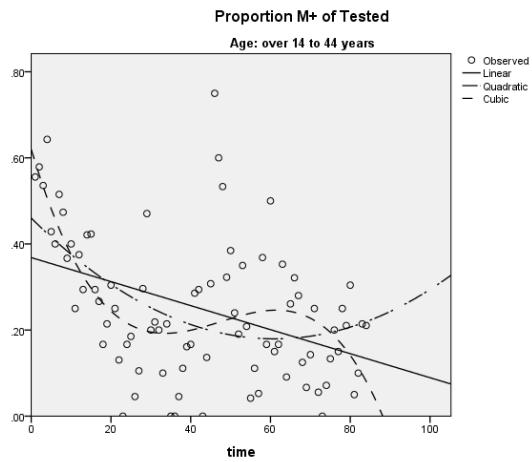
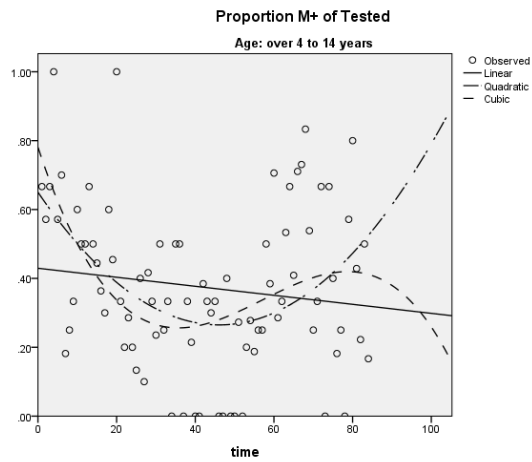
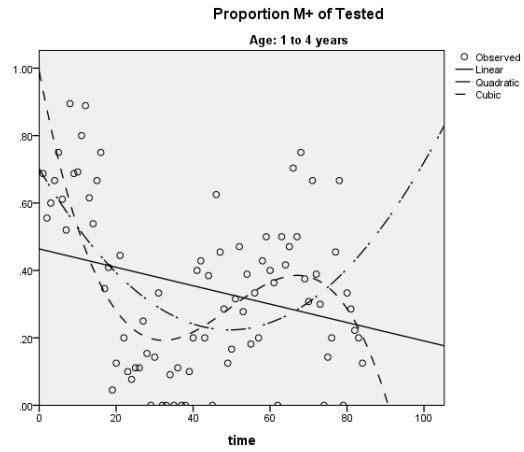
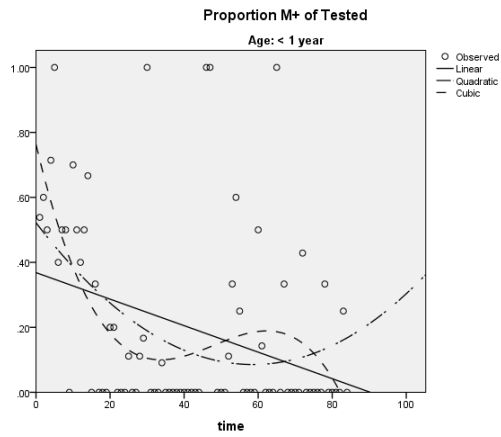
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5	2007	2	4	.50	18	.49	.99	.01
5	2007	4	10	.40	19	.30	.70	.10
5	2007	1	2	.50	20	.69	1.19	-.19
5	2007	0	2	.00	21	.00	.00	.00
5	2007	0	1	.00	22	.00	.00	.00
5	2007	0	3	.00	23	.00	.00	.00
5	2007	2	2	1.00	24	.00	1.00	1.00
5	2008	0	3	.00	25	.00	.00	.00
5	2008	0	2	.00	26	.00	.00	.00
5	2008	0	1	.00	27	.00	.00	.00
5	2008	0	2	.00	28	.00	.00	.00
5	2008	1	2	.50	29	.69	1.19	-.19
5	2008	0	3	.00	30	.00	.00	.00
5	2008	0	3	.00	31	.00	.00	.00
5	2008	0	3	.00	32	.00	.00	.00
5	2008	1	1	1.00	33	.00	1.00	1.00
5	2008	0	2	.00	34	.00	.00	.00
5	2008	0	1	.00	36	.00	.00	.00
5	2009	0	1	.00	37	.00	.00	.00
5	2009	1	11	.09	39	.17	.26	-.08
5	2009	1	3	.33	40	.53	.87	-.20
5	2009	1	1	1.00	41	.00	1.00	1.00
5	2009	0	2	.00	42	.00	.00	.00
5	2009	0	4	.00	43	.00	.00	.00
5	2009	1	4	.25	44	.42	.67	-.17
5	2009	0	4	.00	45	.00	.00	.00
5	2009	0	1	.00	46	.00	.00	.00
5	2009	0	2	.00	47	.00	.00	.00
5	2009	1	3	.33	48	.53	.87	-.20
5	2010	1	8	.13	49	.23	.35	-.10
5	2010	1	2	.50	50	.69	1.19	-.19
5	2010	0	3	.00	51	.00	.00	.00
5	2010	0	4	.00	52	.00	.00	.00
5	2010	1	2	.50	53	.69	1.19	-.19
5	2010	0	3	.00	54	.00	.00	.00
5	2010	1	5	.20	55	.35	.55	-.15
5	2010	1	4	.25	56	.42	.67	-.17
5	2010	0	5	.00	57	.00	.00	.00
5	2010	1	5	.20	58	.35	.55	-.15
5	2010	1	5	.20	59	.35	.55	-.15
5	2010	3	8	.38	60	.34	.71	.04
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5	2011	0	2	.00	62	.00	.00	.00

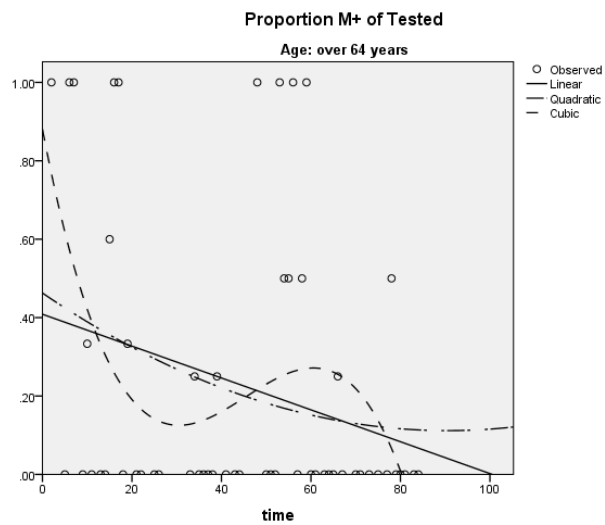
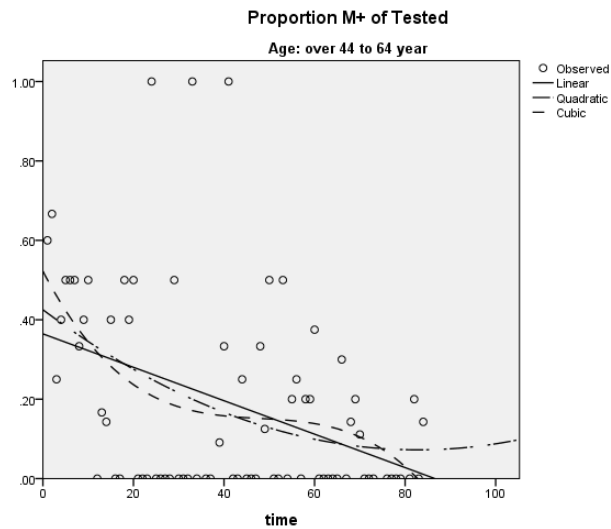
5	2011	0	3	.00	63	.00	.00	.00
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5	2011	0	2	.00	65	.00	.00	.00
5	2011	3	10	.30	66	.28	.58	.02
5	2011	0	1	.00	67	.00	.00	.00
5	2011	1	7	.14	68	.26	.40	-.12
5	2011	1	5	.20	69	.35	.55	-.15
5	2011	1	9	.11	70	.21	.32	-.09
5	2011	0	2	.00	71	.00	.00	.00
5	2011	0	5	.00	72	.00	.00	.00
5	2012	0	4	.00	73	.00	.00	.00
5	2012	0	4	.00	76	.00	.00	.00
5	2012	0	2	.00	77	.00	.00	.00
5	2012	0	2	.00	78	.00	.00	.00
5	2012	0	5	.00	79	.00	.00	.00
5	2012	0	6	.00	81	.00	.00	.00
5	2012	1	5	.20	82	.35	.55	-.15
5	2012	0	8	.00	83	.00	.00	.00
5	2012	1	7	.14	84	.26	.40	-.12
6	2006	1	1	1.00	2	.00	1.00	1.00
6	2006		0		4			
6	2006	0	1	.00	5	.00	.00	.00
6	2006	2	2	1.00	6	.00	1.00	1.00
6	2006	1	1	1.00	7	.00	1.00	1.00
6	2006	0	1	.00	9	.00	.00	.00
6	2006	1	3	.33	10	.53	.87	-.20
6	2006	0	1	.00	11	.00	.00	.00
6	2007	0	5	.00	13	.00	.00	.00
6	2007	0	2	.00	14	.00	.00	.00
6	2007	3	5	.60	15	.43	1.03	.17
6	2007	1	1	1.00	16	.00	1.00	1.00
6	2007	1	1	1.00	17	.00	1.00	1.00
6	2007	0	1	.00	18	.00	.00	.00
6	2007	1	3	.33	19	.53	.87	-.20
6	2007	0	1	.00	21	.00	.00	.00
6	2007	0	1	.00	22	.00	.00	.00
6	2008	0	2	.00	25	.00	.00	.00
6	2008	0	1	.00	26	.00	.00	.00
6	2008	0	2	.00	33	.00	.00	.00
6	2008	1	4	.25	34	.42	.67	-.17
6	2008	0	1	.00	35	.00	.00	.00
6	2008	0	1	.00	36	.00	.00	.00
6	2009	0	1	.00	37	.00	.00	.00
6	2009	0	1	.00	38	.00	.00	.00

6	2009	1	4	.25	39	.42	.67	-.17
6	2009	0	1	.00	41	.00	.00	.00
6	2009	0	1	.00	43	.00	.00	.00
6	2009	0	1	.00	44	.00	.00	.00
6	2009	1	1	1.00	48	.00	1.00	1.00
6	2010	0	1	.00	50	.00	.00	.00
6	2010	0	1	.00	51	.00	.00	.00
6	2010	0	1	.00	52	.00	.00	.00
6	2010	1	1	1.00	53	.00	1.00	1.00
6	2010	1	2	.50	54	.69	1.19	-.19
6	2010	1	2	.50	55	.69	1.19	-.19
6	2010	1	1	1.00	56	.00	1.00	1.00
6	2010	0	1	.00	57	.00	.00	.00
6	2010	1	2	.50	58	.69	1.19	-.19
6	2010	1	1	1.00	59	.00	1.00	1.00
6	2010	0	2	.00	60	.00	.00	.00
6	2011	0	2	.00	61	.00	.00	.00
6	2011	0	2	.00	63	.00	.00	.00
6	2011	0	4	.00	64	.00	.00	.00
6	2011	0	4	.00	65	.00	.00	.00
6	2011	1	4	.25	66	.42	.67	-.17
6	2011	0	3	.00	67	.00	.00	.00
6	2011	0	2	.00	70	.00	.00	.00
6	2011	0	1	.00	71	.00	.00	.00
6	2012	0	1	.00	73	.00	.00	.00
6	2012	0	1	.00	75	.00	.00	.00
6	2012	0	3	.00	77	.00	.00	.00
6	2012	1	2	.50	78	.69	1.19	-.19
6	2012	0	2	.00	79	.00	.00	.00
6	2012	0	1	.00	80	.00	.00	.00
6	2012	0	1	.00	81	.00	.00	.00
6	2012	0	4	.00	83	.00	.00	.00
6	2012	0	3	.00	84	.00	.00	.00

Age Group	Trend	R-Squared	Regression significance
<1	Linear	.116	.002
	Quadratic	.168	.001

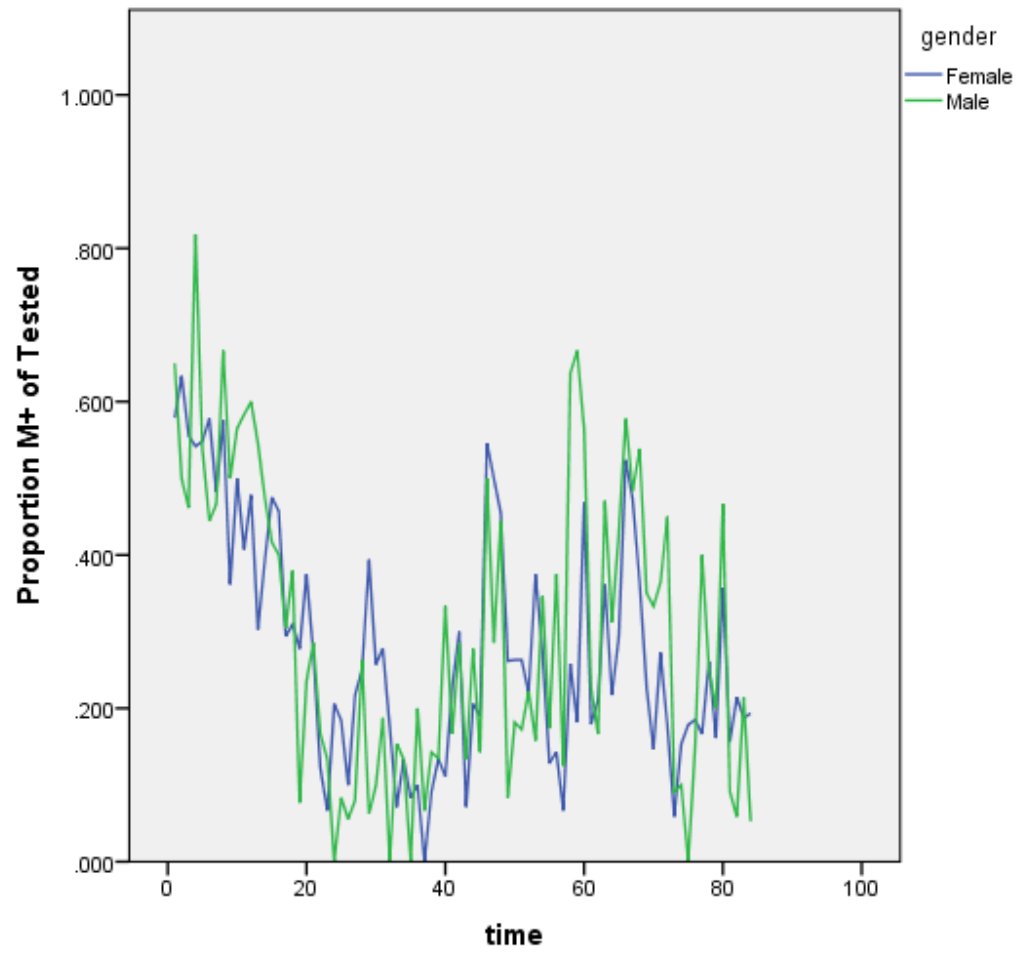
1 - 4	Cubic	.254	<0.001
	Linear	.074	.012
	Quadratic	.252	<0.001
5 - 14	Cubic	.428	<0.001
	Linear	.018	.223
	Quadratic	.18	<0.001
15 - 44	Cubic	.218	<0.001
	Linear	.169	<0.001
	Quadratic	.277	<0.001
45 – 64	Cubic	.344	<0.001
	Linear	.159	<0.001
	Quadratic	.170	.001
≥65	Cubic	.190	.001
	Linear	.071	.045
	Quadratic	.075	.122
	Cubic	.171	.018

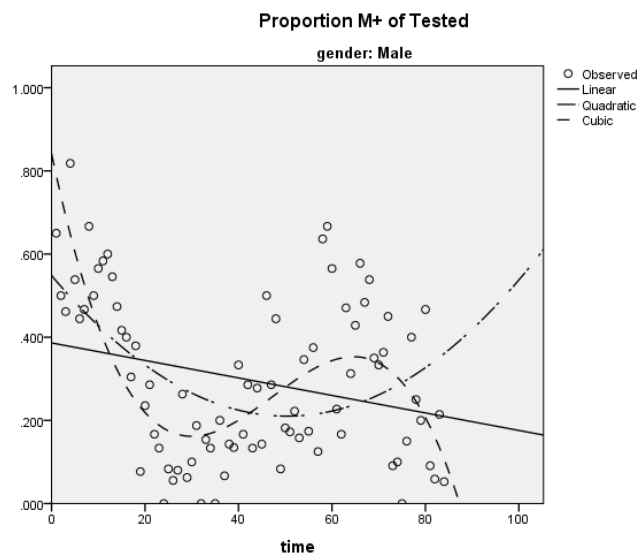
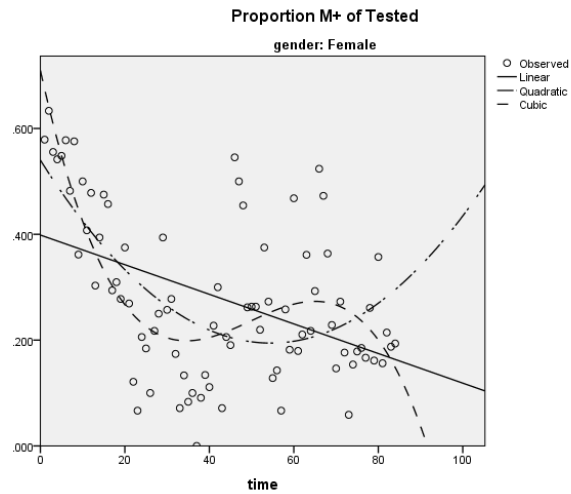




Year x Month Trend (...within gender groups)

Age Group	Trend	R-Squared	Regression significance
Female (1)	Linear	.197	<0.001
	Quadratic	.356	<0.001
	Cubic	.508	<0.001
Male (2)	Linear	.069	.016
	Quadratic	.198	<0.001
	Cubic	.477	<0.001





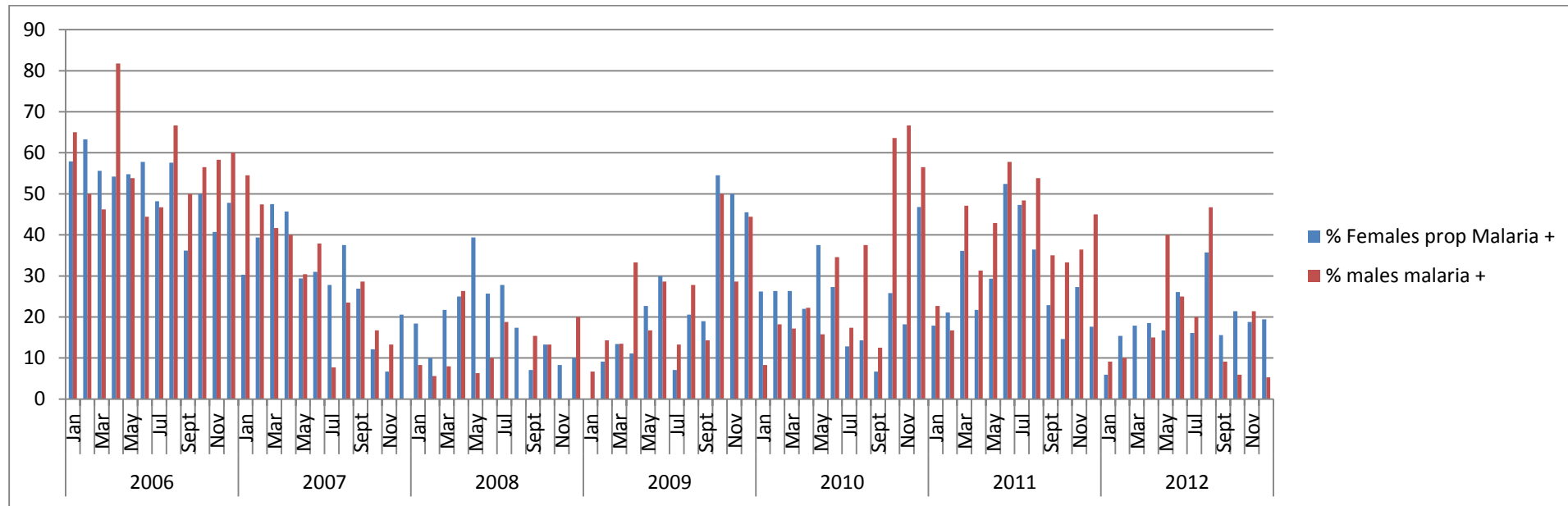
gender	Year	CNT_POS	CNT_TST	proppos	time	CI_Factor	CI95_Upper	CI95_Lower
1	2006	22	38	.579	1	.16	.74	.42
1	2006	19	30	.633	2	.17	.81	.46
1	2006	25	45	.556	3	.15	.70	.41
1	2006	13	24	.542	4	.20	.74	.34
1	2006	17	31	.548	5	.18	.72	.37
1	2006	26	45	.578	6	.14	.72	.43
1	2006	27	56	.482	7	.13	.61	.35
1	2006	19	33	.576	8	.17	.74	.41
1	2006	17	47	.362	9	.14	.50	.22
1	2006	21	42	.500	10	.15	.65	.35
1	2006	11	27	.407	11	.19	.59	.22
1	2006	11	23	.478	12	.20	.68	.27
1	2007	10	33	.303	13	.16	.46	.15
1	2007	13	33	.394	14	.17	.56	.23
1	2007	19	40	.475	15	.15	.63	.32

1	2007	16	35	.457	16	.17	.62	.29
1	2007	10	34	.294	17	.15	.45	.14
1	2007	13	42	.310	18	.14	.45	.17
1	2007	15	54	.278	19	.12	.40	.16
1	2007	9	24	.375	20	.19	.57	.18
1	2007	7	26	.269	21	.17	.44	.10
1	2007	4	33	.121	22	.11	.23	.01
1	2007	1	15	.067	23	.13	.19	-.06
1	2007	7	34	.206	24	.14	.34	.07
1	2008	7	38	.184	25	.12	.31	.06
1	2008	3	30	.100	26	.11	.21	-.01
1	2008	5	23	.217	27	.17	.39	.05
1	2008	11	44	.250	28	.13	.38	.12
1	2008	13	33	.394	29	.17	.56	.23
1	2008	9	35	.257	30	.14	.40	.11
1	2008	10	36	.278	31	.15	.42	.13
1	2008	4	23	.174	32	.15	.33	.02
1	2008	1	14	.071	33	.13	.21	-.06
1	2008	4	30	.133	34	.12	.25	.01
1	2008	1	12	.083	35	.16	.24	-.07
1	2008	2	20	.100	36	.13	.23	-.03
1	2009	0	30	.000	37	.00	.00	.00
1	2009	1	11	.091	38	.17	.26	-.08
1	2009	13	97	.134	39	.07	.20	.07
1	2009	3	27	.111	40	.12	.23	-.01
1	2009	5	22	.227	41	.18	.40	.05
1	2009	9	30	.300	42	.16	.46	.14
1	2009	2	28	.071	43	.10	.17	-.02
1	2009	7	34	.206	44	.14	.34	.07
1	2009	4	21	.190	45	.17	.36	.02
1	2009	6	11	.545	46	.29	.84	.25
1	2009	7	14	.500	47	.26	.76	.24
1	2009	10	22	.455	48	.21	.66	.25
1	2010	11	42	.262	49	.13	.39	.13
1	2010	5	19	.263	50	.20	.46	.07
1	2010	10	38	.263	51	.14	.40	.12
1	2010	9	41	.220	52	.13	.35	.09
1	2010	15	40	.375	53	.15	.53	.22
1	2010	12	44	.273	54	.13	.40	.14
1	2010	5	39	.128	55	.10	.23	.02
1	2010	2	14	.143	56	.18	.33	-.04
1	2010	2	30	.067	57	.09	.16	-.02
1	2010	8	31	.258	58	.15	.41	.10
1	2010	6	33	.182	59	.13	.31	.05

1	2010	22	47	.468	60	.14	.61	.33
1	2011	7	39	.179	61	.12	.30	.06
1	2011	4	19	.211	62	.18	.39	.03
1	2011	13	36	.361	63	.16	.52	.20
1	2011	5	23	.217	64	.17	.39	.05
1	2011	12	41	.293	65	.14	.43	.15
1	2011	33	63	.524	66	.12	.65	.40
1	2011	26	55	.473	67	.13	.60	.34
1	2011	12	33	.364	68	.16	.53	.20
1	2011	8	35	.229	69	.14	.37	.09
1	2011	6	41	.146	70	.11	.25	.04
1	2011	6	22	.273	71	.19	.46	.09
1	2011	6	34	.176	72	.13	.30	.05
1	2012	2	34	.059	73	.08	.14	-.02
1	2012	2	13	.154	74	.20	.35	-.04
1	2012	5	28	.179	75	.14	.32	.04
1	2012	5	27	.185	76	.15	.33	.04
1	2012	6	36	.167	77	.12	.29	.04
1	2012	6	23	.261	78	.18	.44	.08
1	2012	5	31	.161	79	.13	.29	.03
1	2012	10	28	.357	80	.18	.53	.18
1	2012	5	32	.156	81	.13	.28	.03
1	2012	6	28	.214	82	.15	.37	.06
1	2012	6	32	.188	83	.14	.32	.05
1	2012	6	31	.194	84	.14	.33	.05
2	2006	13	20	.650	1	.21	.86	.44
2	2006	7	14	.500	2	.26	.76	.24
2	2006	12	26	.462	3	.19	.65	.27
2	2006	18	22	.818	4	.16	.98	.66
2	2006	7	13	.538	5	.27	.81	.27
2	2006	8	18	.444	6	.23	.67	.21
2	2006	14	30	.467	7	.18	.65	.29
2	2006	12	18	.667	8	.22	.88	.45
2	2006	11	22	.500	9	.21	.71	.29
2	2006	13	23	.565	10	.20	.77	.36
2	2006	7	12	.583	11	.28	.86	.30
2	2006	6	10	.600	12	.30	.90	.30
2	2007	12	22	.545	13	.21	.75	.34
2	2007	9	19	.474	14	.22	.70	.25
2	2007	5	12	.417	15	.28	.70	.14
2	2007	4	10	.400	16	.30	.70	.10
2	2007	7	23	.304	17	.19	.49	.12
2	2007	11	29	.379	18	.18	.56	.20
2	2007	2	26	.077	19	.10	.18	-.03

2	2007	4	17	.235	20	.20	.44	.03
2	2007	4	14	.286	21	.24	.52	.05
2	2007	3	18	.167	22	.17	.34	-.01
2	2007	2	15	.133	23	.17	.31	-.04
2	2007	0	10	.000	24	.00	.00	.00
2	2008	3	36	.083	25	.09	.17	-.01
2	2008	1	18	.056	26	.11	.16	-.05
2	2008	2	25	.080	27	.11	.19	-.03
2	2008	5	19	.263	28	.20	.46	.07
2	2008	1	16	.063	29	.12	.18	-.06
2	2008	2	20	.100	30	.13	.23	-.03
2	2008	3	16	.188	31	.19	.38	.00
2	2008	0	6	.000	32	.00	.00	.00
2	2008	2	13	.154	33	.20	.35	-.04
2	2008	2	15	.133	34	.17	.31	-.04
2	2008	0	8	.000	35	.00	.00	.00
2	2008	2	10	.200	36	.25	.45	-.05
2	2009	1	15	.067	37	.13	.19	-.06
2	2009	1	7	.143	38	.26	.40	-.12
2	2009	7	52	.135	39	.09	.23	.04
2	2009	3	9	.333	40	.31	.64	.03
2	2009	2	12	.167	41	.21	.38	-.04
2	2009	4	14	.286	42	.24	.52	.05
2	2009	2	15	.133	43	.17	.31	-.04
2	2009	5	18	.278	44	.21	.48	.07
2	2009	1	7	.143	45	.26	.40	-.12
2	2009	4	8	.500	46	.35	.85	.15
2	2009	2	7	.286	47	.33	.62	-.05
2	2009	4	9	.444	48	.32	.77	.12
2	2010	1	12	.083	49	.16	.24	-.07
2	2010	2	11	.182	50	.23	.41	-.05
2	2010	5	29	.172	51	.14	.31	.03
2	2010	4	18	.222	52	.19	.41	.03
2	2010	3	19	.158	53	.16	.32	-.01
2	2010	9	26	.346	54	.18	.53	.16
2	2010	4	23	.174	55	.15	.33	.02
2	2010	3	8	.375	56	.34	.71	.04
2	2010	1	8	.125	57	.23	.35	-.10
2	2010	7	11	.636	58	.28	.92	.35
2	2010	6	9	.667	59	.31	.97	.36
2	2010	13	23	.565	60	.20	.77	.36
2	2011	5	22	.227	61	.18	.40	.05
2	2011	3	18	.167	62	.17	.34	-.01
2	2011	8	17	.471	63	.24	.71	.23

2	2011	5	16	.313	64	.23	.54	.09
2	2011	12	28	.429	65	.18	.61	.25
2	2011	26	45	.578	66	.14	.72	.43
2	2011	15	31	.484	67	.18	.66	.31
2	2011	7	13	.538	68	.27	.81	.27
2	2011	7	20	.350	69	.21	.56	.14
2	2011	5	15	.333	70	.24	.57	.09
2	2011	4	11	.364	71	.28	.65	.08
2	2011	9	20	.450	72	.22	.67	.23
2	2012	1	11	.091	73	.17	.26	-.08
2	2012	1	10	.100	74	.19	.29	-.09
2	2012	0	6	.000	75	.00	.00	.00
2	2012	3	20	.150	76	.16	.31	-.01
2	2012	4	10	.400	77	.30	.70	.10
2	2012	2	8	.250	78	.30	.55	-.05
2	2012	3	15	.200	79	.20	.40	.00
2	2012	7	15	.467	80	.25	.72	.21
2	2012	1	11	.091	81	.17	.26	-.08
2	2012	1	17	.059	82	.11	.17	-.05
2	2012	3	14	.214	83	.21	.43	.00
2	2012	1	19	.053	84	.10	.15	-.05



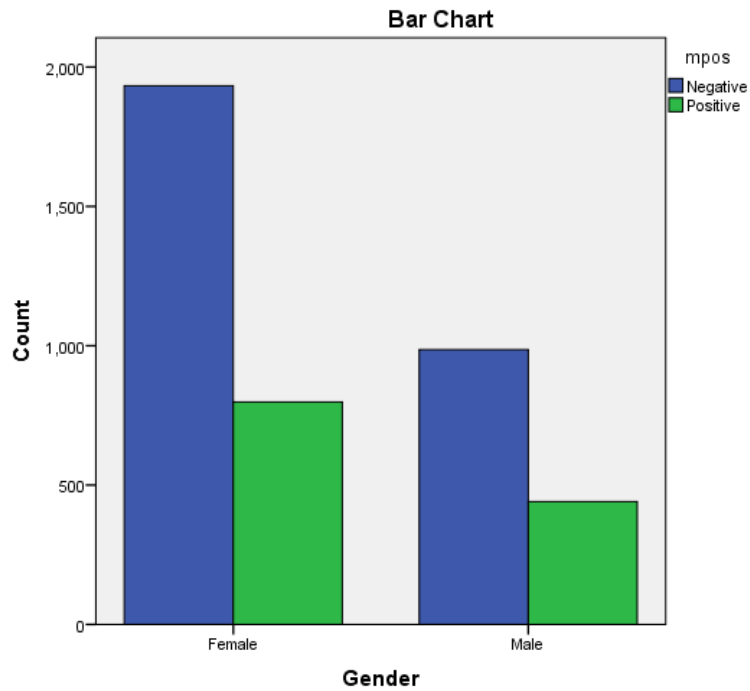
Prevalence X Gender

Statistic	Value
Pearson Chi-Square (p-value)	.260
Phi	.017
Cramer's V	.017
Cells with expected count less than 5 (%)	0

ODDS

	Negative	Positive	Odds Malaria +
Female	1933	798	0.412829798
Male	986	441	0.447261663

Odds ratio for Malaria females compared to males= 1.083404505
 Thus an slightly increased risk of malaria for males
 SE ln(OR)= 0.07107908
 CI Upper 1.24535845
 CI Lower 0.94251203
 CI does not contain zero therefore OR is statistically significant
 However, OR is only slightly above 1 and therefore not practically significant



Gender	CNT_POS	CNT_TST	proppos	CI_Factor	CI95_Upper	CI95_Lower
F	798	2731	.292	.02	.31	.28
M	441	1427	.309	.02	.33	.29

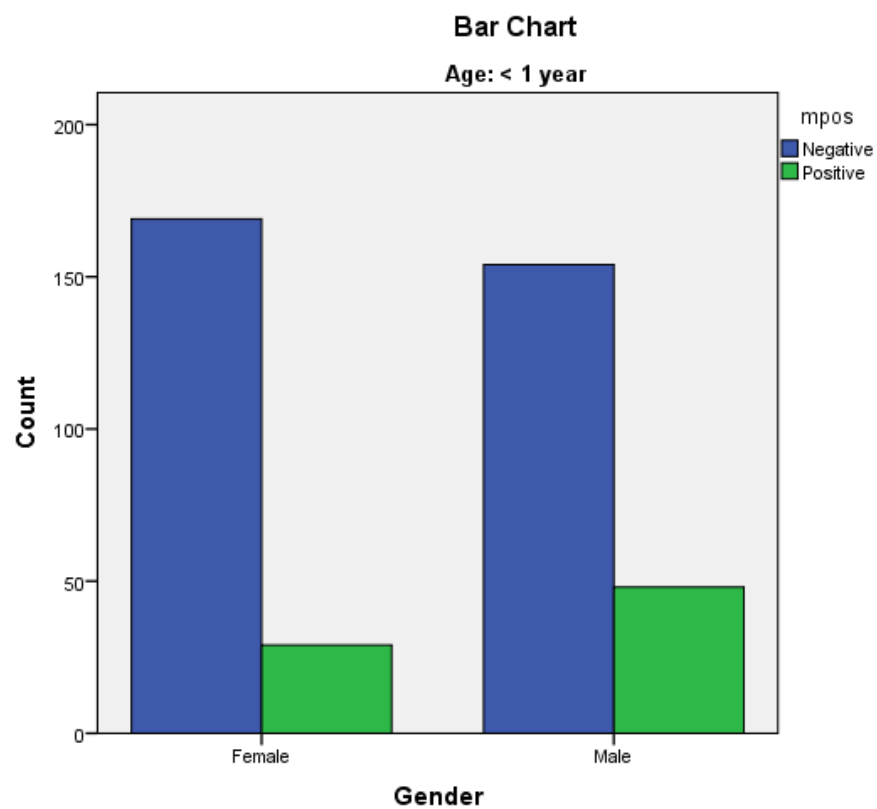
SPLIT BY AGE

<1

Statistic	Value
Pearson Chi-Square (p-value)	.021
Phi	.116
Cramer's V	.116
Cells with expected count less than 5 (%)	0

ODDS			
	Negative	Positive	Odds Malaria +
Female	169	29	0.171597633
Male	154	48	0.311688312

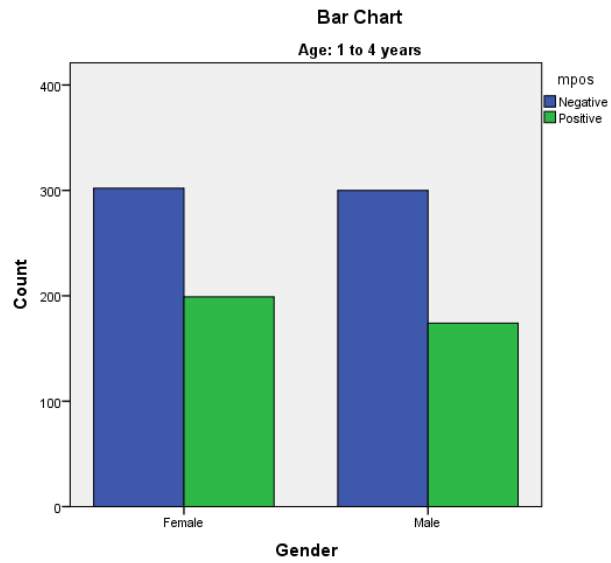
Odds ratio for Malaria females compared to males= 1.816391
 Thus an slightly increased risk of malaria for males
 SE ln(OR)= 0.260244
 CI Upper 3.025054
 CI Lower 1.09065
 CI does not contain zero therefore OR is statistically significant
 However, OR is only slightly above 1 and therefore not practically significant



1 - 4

Statistic	Value
Pearson Chi-Square (p-value)	.333
Phi	.031
Cramer's V	.031
Cells with expected count less than 5 (%)	0

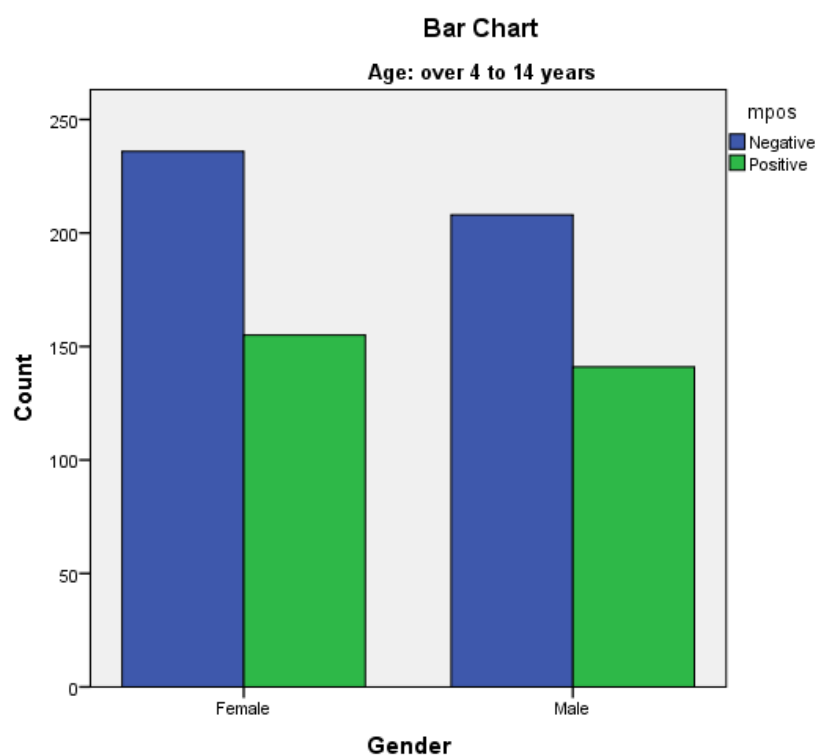
ODDS				
	Negative	Positive	Odds Malaria +	
Female	302	199	0.658940397	
Male	300	174	0.58	
Odds ratio for Malaria females compared to males=				1.136104
Thus an slightly increased risk of malaria for females				
SE ln(OR)=	0.131973			
CI Upper	1.471485			
CI Lower	0.877163			
CI does not contain zero therefore OR is statistically significant				
However, OR is only slightly above 1 and therefore not practically significant				



5 - 14

Statistic	Value
Pearson Chi-Square (p-value)	.833
Phi	.008
Cramer's V	.008
Cells with expected count less than 5 (%)	0

ODDS			
	Negative	Positive	Odds Malaria +
Female	236	155	0.656779661
Male	208	141	0.677884615
Odds ratio for Malaria females compared to males= 1.032134 Thus an slightly increased risk of malaria for males SE ln(OR)= 0.150296 CI Upper 1.385704 CI Lower 0.76878 CI does not contain zero therefore OR is statistically significant However, OR is only slightly above 1 and therefore not practically significant			



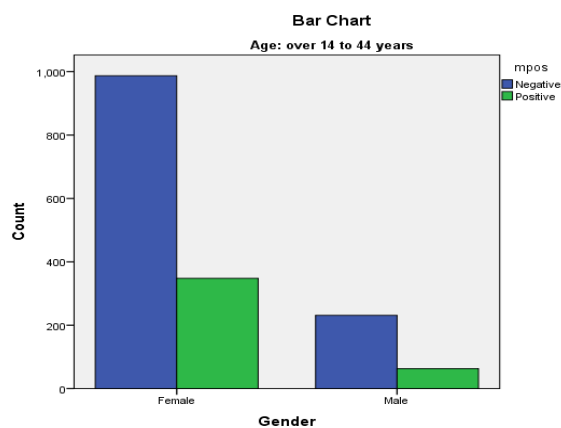
15 - 44

Statistic	Value
Pearson Chi-Square (p-value)	.097
Phi	.041

Cramer's V .041

Cells with expected count less than 5 (%) 0

ODDS			
	Negative	Positive	Odds Malaria +
Female	987	348	0.352583587
Male	231	63	0.272727273
Odds ratio for Malaria females compared to males=			1.292806
Thus an slightly increased risk of malaria for females			
SE ln(OR)=	0.155206		
CI Upper	1.752456		
CI Lower	0.953718		
CI does not contain zero therefore OR is statistically significant			
However, OR is only slightly above 1 and therefore not practically significant			

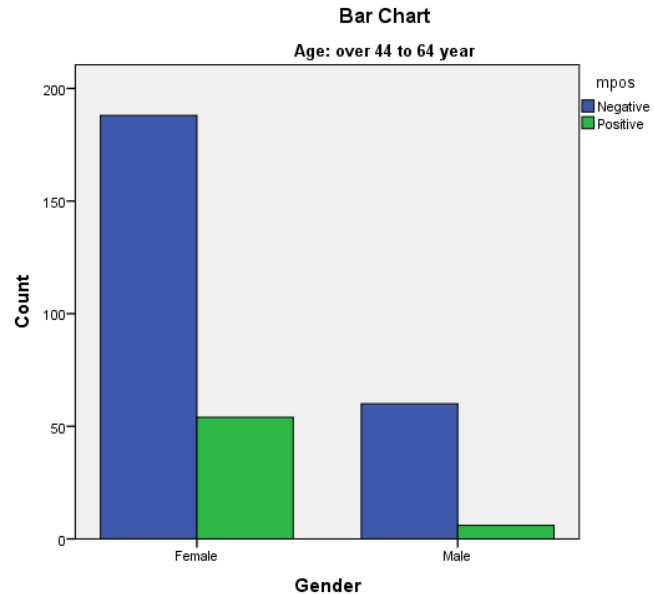


45 – 64

Statistic	Value
Pearson Chi-Square (p-value)	.016
Phi	.137
Cramer's V	.137
Cells with expected count less than 5 (%)	0

ODDS			
	Negative	Positive	Odds Malaria +
Female	188	54	0.287234043
Male	60	6	0.1

Odds ratio for Malaria females compared to males= 2.87234
 Thus an increased risk of malaria for females
 SE ln(OR)= 0.45516
 CI Upper 7.009326
 CI Lower 1.177052
 CI does not contain zero therefore OR is statistically significant
 OR is practically visible



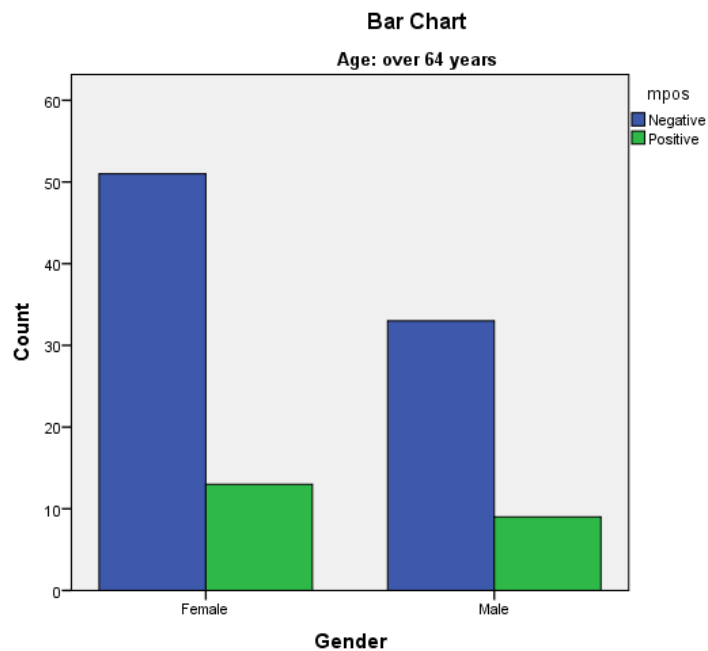
≥65

Statistic

Value

Pearson Chi-Square (p-value)	.98
Phi	.013
Cramer's V	.013
Cells with expected count less than 5 (%)	0

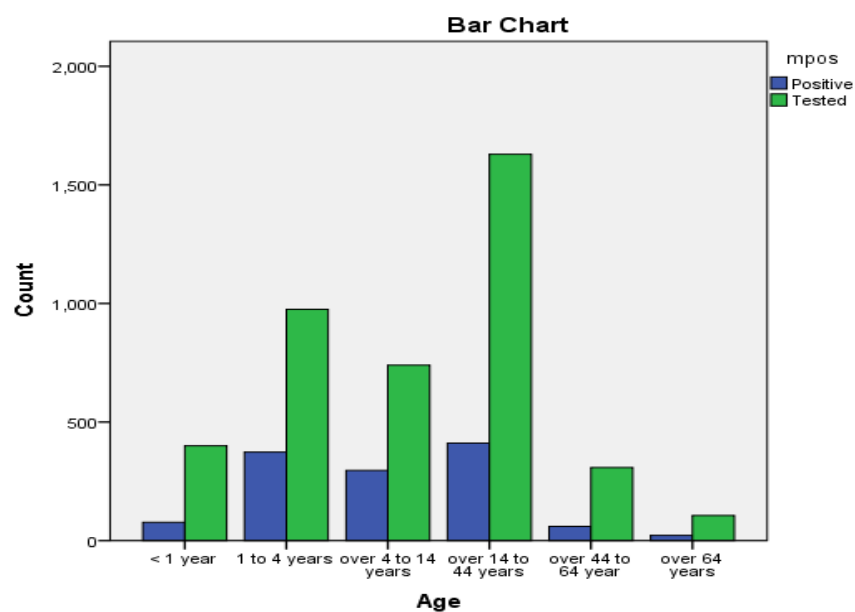
ODDS				
	Negative	Positive	Odds Malaria +	
Female	51	13	0.254901961	
Male	33	9	0.272727273	
Odds ratio for Malaria females compared to males=				1.06993
Thus a slightly increased risk of malaria for males				
SE ln(OR)=	0.487796			
CI Upper	2.783401			
CI Lower	0.411277			
CI does not contain zero therefore OR is statistically significant				
OR is not practically significant				



< 1 year	Count
1 to 4 years	Count
over 4 to 14 years	Count
over 14 to 44 years	Count
over 44 to 64 year	Count
over 64 years	Count

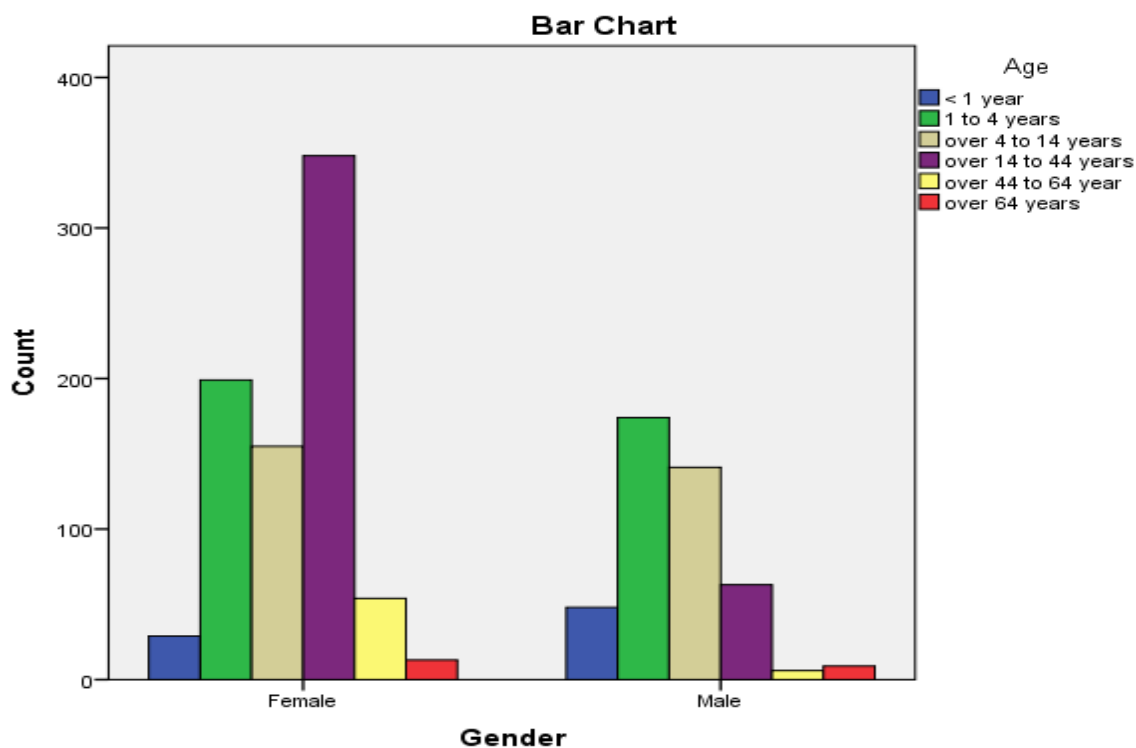
mpos	
Positive	Tested
77 _a	400 _b
373 _a	975 _b
296 _a	740 _b
411 _a	1629 _b
60 _a	308 _b
22 _a	106 _a

Statistic	Value
Pearson Chi-Square (p-value)	<.001
Likelihood Ratio (p-value)	<.001
Linear-by-Linear Association (p-value)	.001
Phi	.113
Cramer's V	.113
Cells with expected count less than 5 (%)	0



Age	CNT_POS	CNT_TST	Proportion M+ of Tested	CI_Factor	CI95_Upper	CI95_Lower
1	77	400	.193	.04	.23	.15
2	373	975	.383	.03	.41	.35
3	296	740	.400	.04	.44	.36
4	411	1629	.252	.02	.27	.23
5	60	308	.195	.04	.24	.15
6	22	106	.208	.08	.28	.13

Age X Gender (M+)



Statistic	Value
Pearson Chi-Square (p-value)	<.001

Likelihood Ratio (p-value)	<.001
Linear-by-Linear Association (p-value)	<.001
Phi	.352
Cramer's V	.352
Cells with expected count less than 5 (%)	0

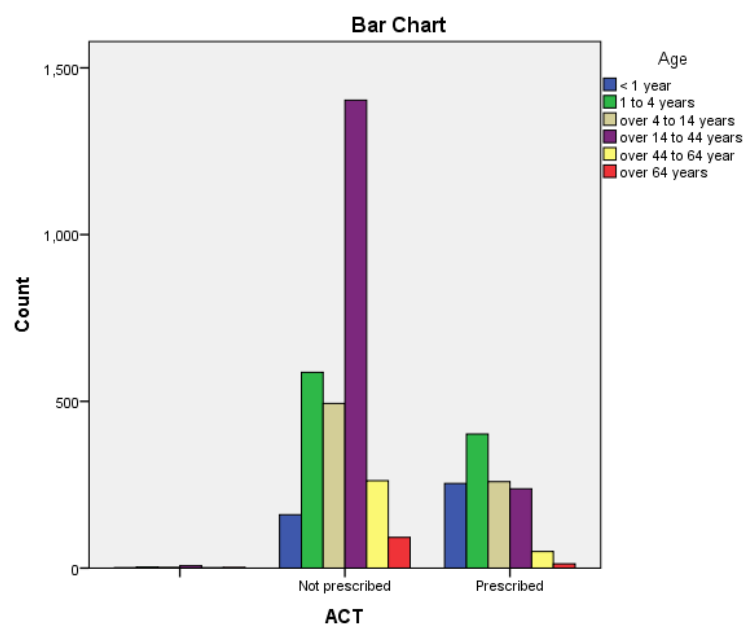
ANNEXSURE 5

Malaria Case Management Results

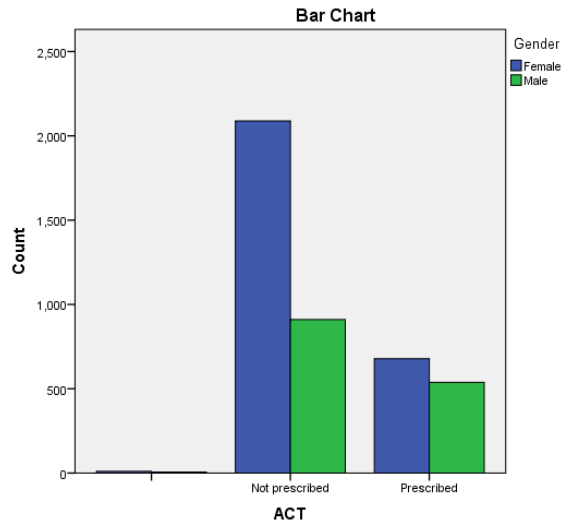
Malaria treatment results

TREATMENT X AGE & GENDER

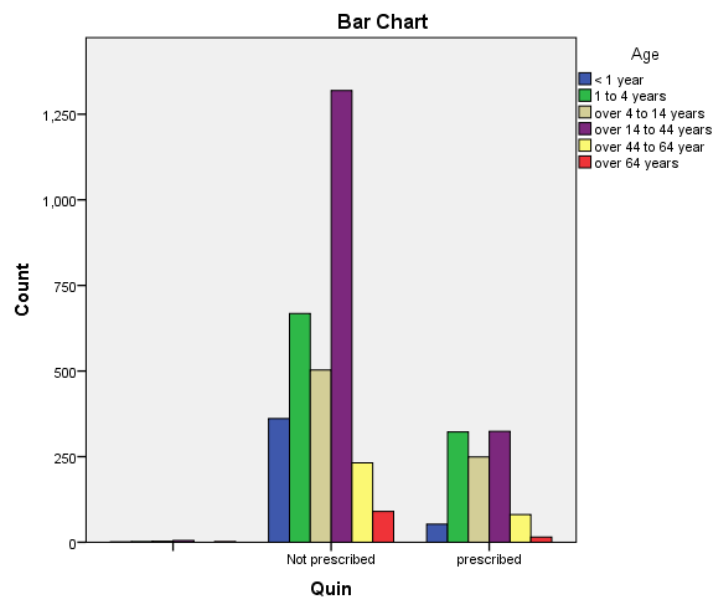
TREATMENT X AGE & GENDER



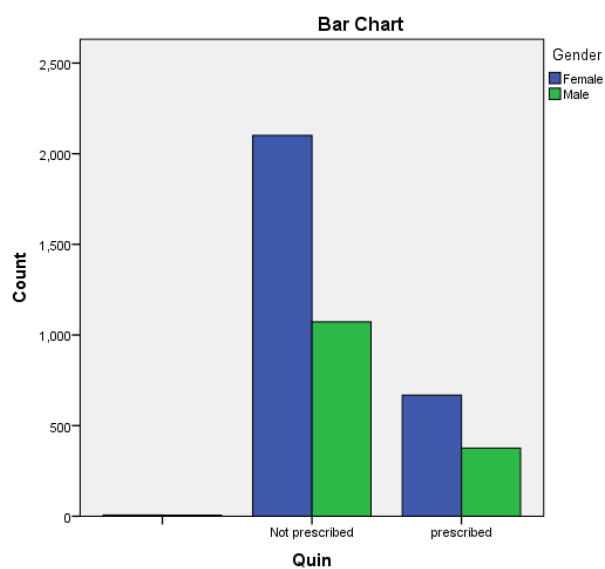
Statistic	Value
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	
Phi	.344
Cramer's V	.244
Cells with expected count less than 5 (%)	27.8



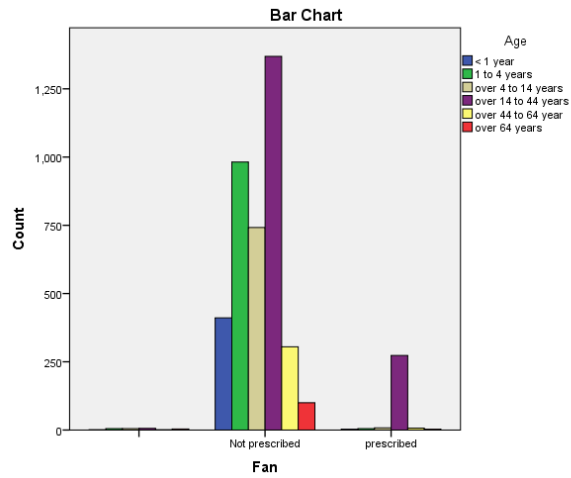
Statistic	Value
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	
Phi	.132
Cramer's V	.132
Cells with expected count less than 5 (%)	0



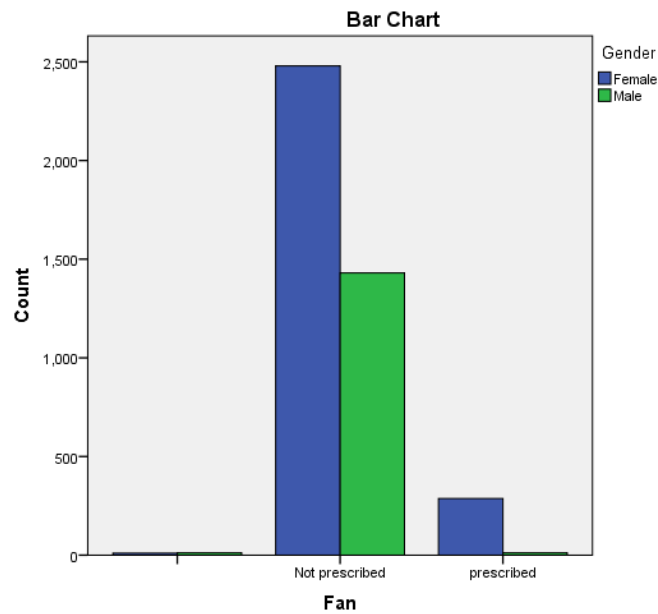
Statistic	Value
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	
Phi	.176
Cramer's V	.124
Cells with expected count less than 5 (%)	27.8



Statistic	Value
Pearson Chi-Square (p-value)	.282
Likelihood Ratio (p-value)	.287
Linear-by-Linear Association (p-value)	
Phi	.024
Cramer's V	.024
Cells with expected count less than 5 (%)	16.7

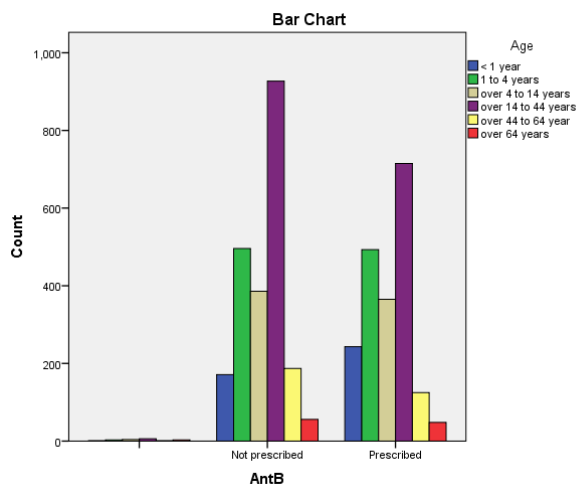


Statistic	Value
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	
Phi	.306
Cramer's V	.216
Cells with expected count less than 5 (%)	22.2

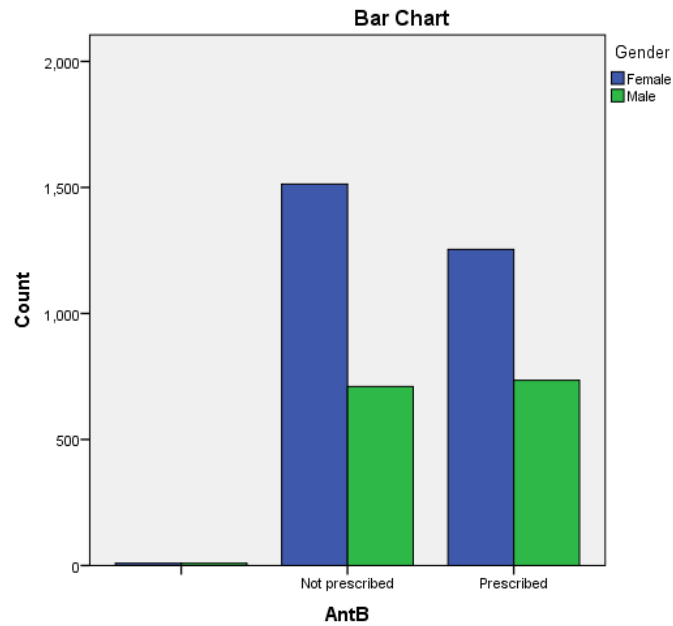


Statistic	Value
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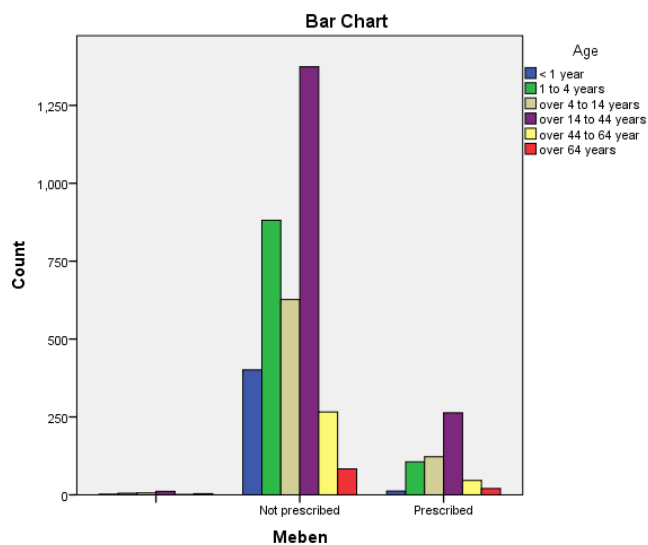
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	
Phi	.178
Cramer's V	.178
Cells with expected count less than 5 (%)	0



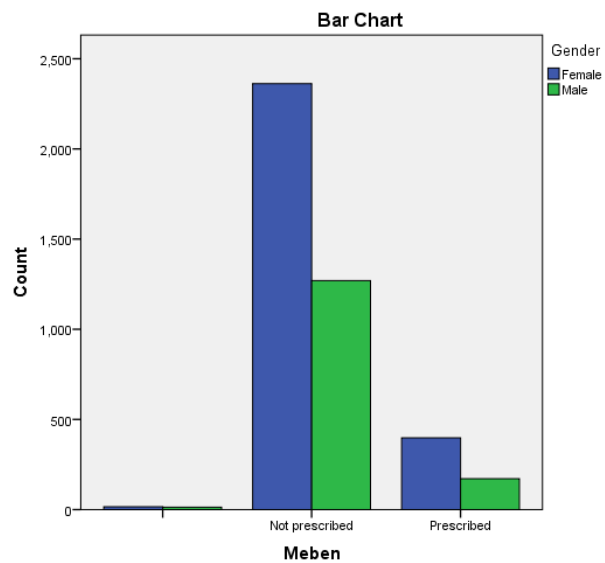
Statistic	Value
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	
Phi	.115
Cramer's V	.081
Cells with expected count less than 5 (%)	27.8



Statistic	Value
Pearson Chi-Square (p-value)	.001
Likelihood Ratio (p-value)	.001
Linear-by-Linear Association (p-value)	
Phi	.057
Cramer's V	.057
Cells with expected count less than 5 (%)	0



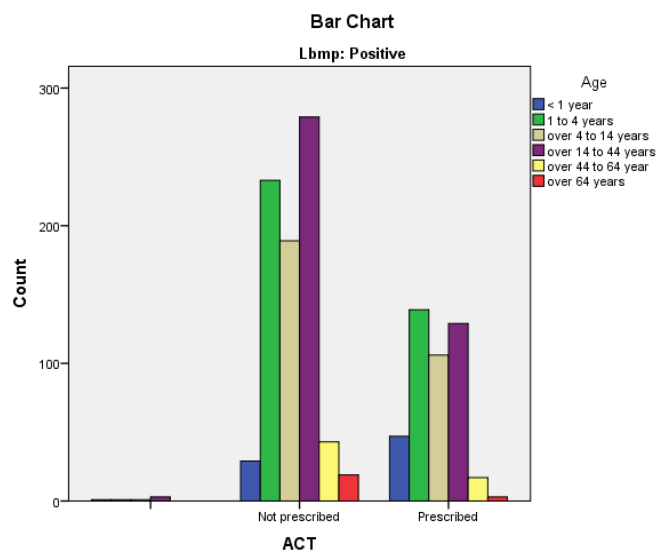
Statistic	Value
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	
Phi	.137
Cramer's V	.097
Cells with expected count less than 5 (%)	16.7



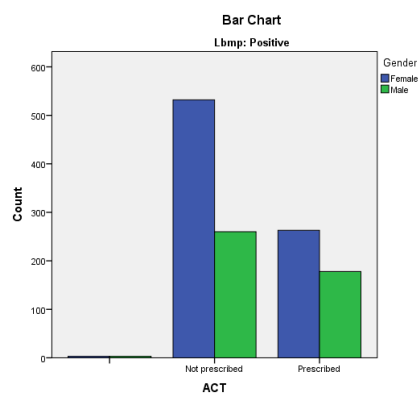
Statistic	Value
Pearson Chi-Square (p-value)	.035
Likelihood Ratio (p-value)	.035
Linear-by-Linear Association (p-value)	
Phi	.040
Cramer's V	.040
Cells with expected count less than 5 (%)	0

TREATMENT X AGE & GENDER (MALARIA +)

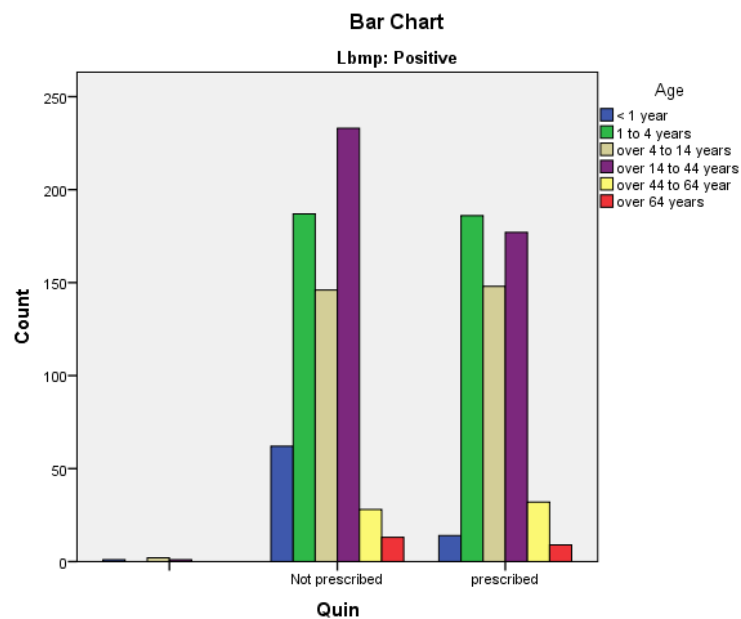
TREATMENT X Age & Gender (MALARIA +)



Statistic	Value
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	
Phi	.167
Cramer's V	.118
Cells with expected count less than 5 (%)	33.3



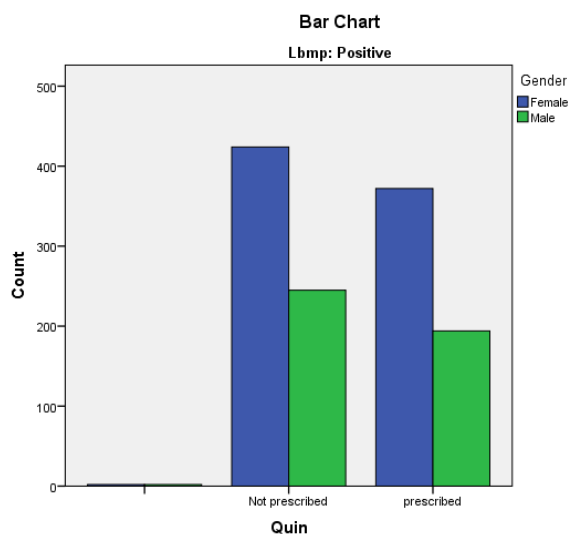
Statistic	Value
Pearson Chi-Square (p-value)	.023
Likelihood Ratio (p-value)	.024
Linear-by-Linear Association (p-value)	
Phi	.078
Cramer's V	.078
Cells with expected count less than 5 (%)	33.3



Statistic	Value
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	
Phi	.169
Cramer's V	.119

Cells with expected count less than 5 (%)

33.3



Statistic

Value

Pearson Chi-Square (p-value)

.577

Likelihood Ratio (p-value)

.581

Linear-by-Linear Association (p-value)

Phi

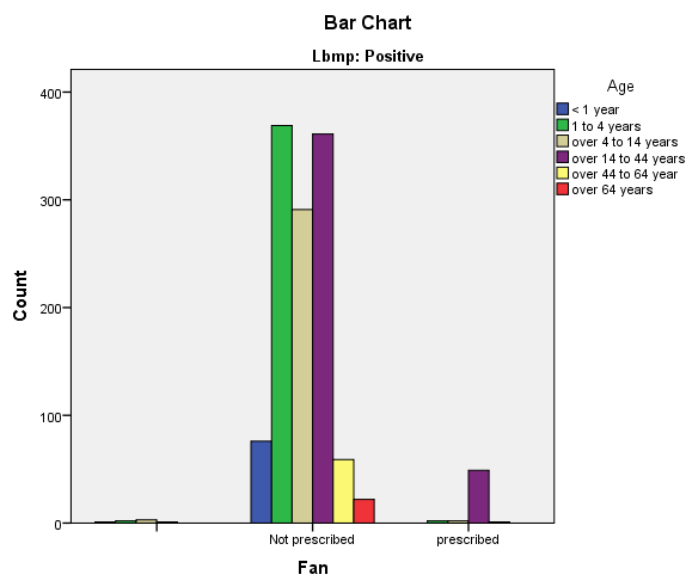
.03

Cramer's V

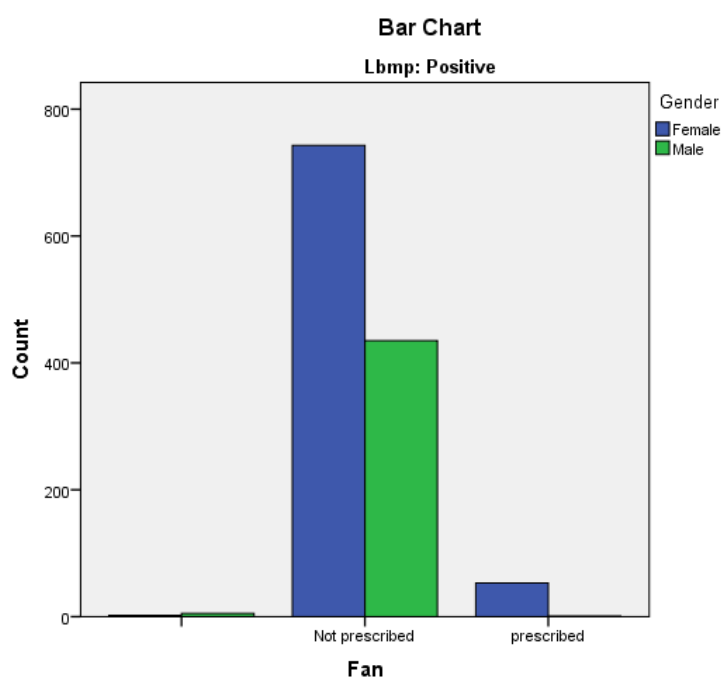
.03

Cells with expected count less than 5 (%)

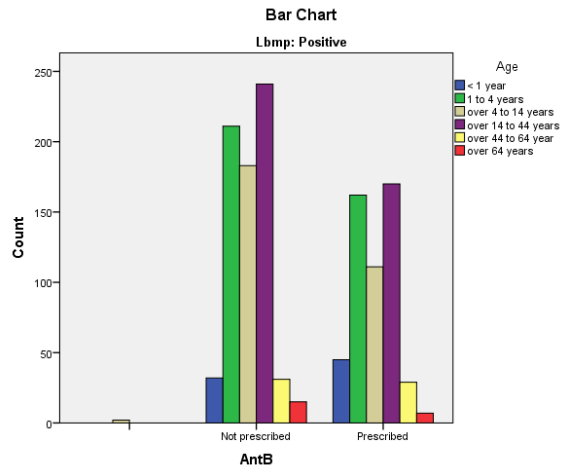
33.3



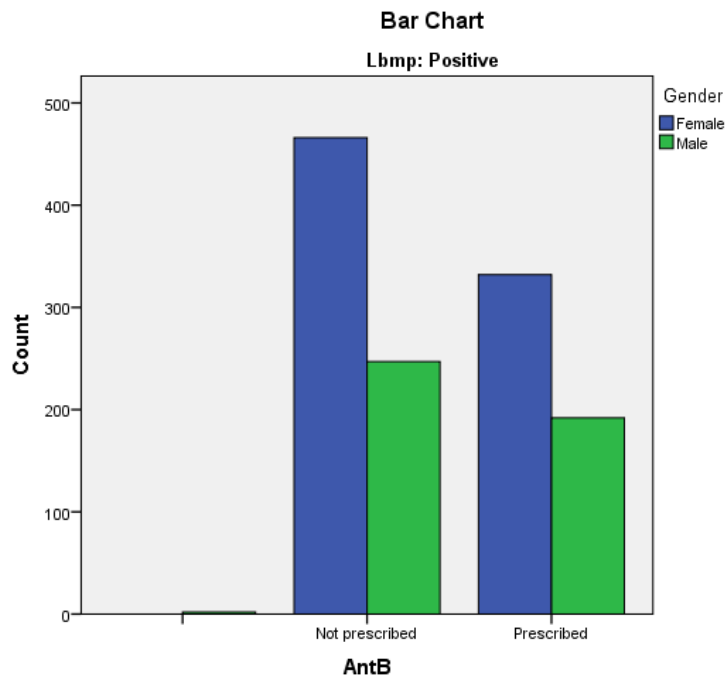
Statistic	Value
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	
Phi	.266
Cramer's V	.188
Cells with expected count less than 5 (%)	50



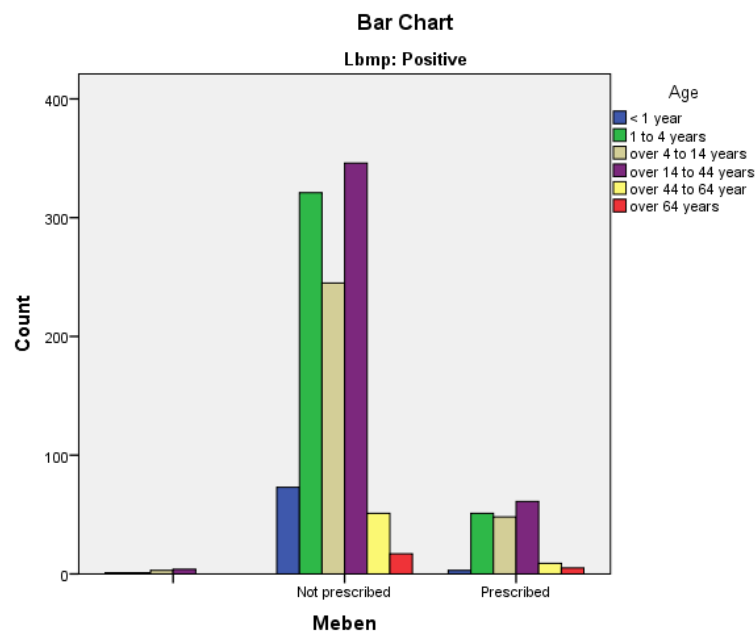
Statistic	Value
Pearson Chi-Square (p-value)	<0.001
Likelihood Ratio (p-value)	<0.001
Linear-by-Linear Association (p-value)	
Phi	.160
Cramer's V	.160
Cells with expected count less than 5 (%)	33.3



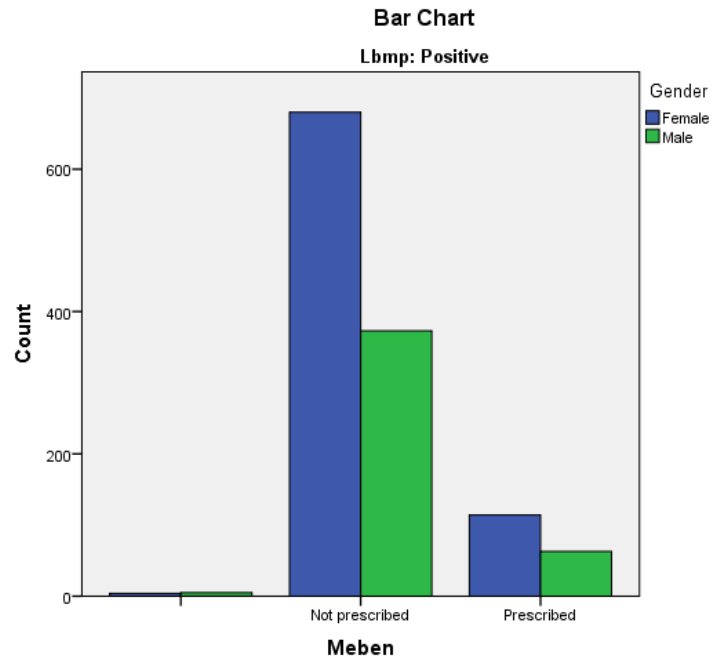
Statistic	Value
Pearson Chi-Square (p-value)	.036
Likelihood Ratio (p-value)	.046
Linear-by-Linear Association (p-value)	
Phi	.125
Cramer's V	.088
Cells with expected count less than 5 (%)	33.3



Statistic	Value
Pearson Chi-Square (p-value)	.125
Likelihood Ratio (p-value)	.097
Linear-by-Linear Association (p-value)	
Phi	.058
Cramer's V	.058
Cells with expected count less than 5 (%)	33.3



Statistic	Value
Pearson Chi-Square (p-value)	.293
Likelihood Ratio (p-value)	.138
Linear-by-Linear Association (p-value)	
Phi	.098
Cramer's V	.069
Cells with expected count less than 5 (%)	38.9



Statistic	Value
Pearson Chi-Square (p-value)	.454
Likelihood Ratio (p-value)	.473
Linear-by-Linear Association (p-value)	
Phi	.036
Cramer's V	.036
Cells with expected count less than 5 (%)	16.7