Chloroquine as a therapeutic option for mild post malaria anaemia

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A Thesis submitted for the degree of Doctor of Philosophy

University of Glasgow

Division of Developmental Medicine

December 2010

1 Acknowledgements

I want to first of all thank God for His grace, and for granting me the health, presence of mind and courage needed to go through this training programme successfully. I would also like to thank my wife Mrs Bamidele Rachael Nweneka, my children Blessing, Christian, Lovely and Delight for their patience, love, encouragement and prayers, and for tolerating my frequent long absences during this process.

My sincere gratitude also goes to Professor Andrew Prentice, Director of the MRC International Nutrition Group, London School of Hygiene and Tropical Medicine for his financial and moral support without which this work would not have been possible. I want to thank my supervisors – Dr Conor Doherty, Professor Lawrence Weaver, and Professor Andrew Prentice for their patience, guidance, support and encouragement from the conception of this project to the final phase of the dissertation. I also want to thank Dr Sophie Moore, the Head of Station of the Keneba Field Station of the MRC, The Gambia and the Management of the MRC International Nutrition Group, Keneba, The Gambia for their financial, and logistic support for this project. I want to thank Dr Sharon Cox for her technical advice and support during the different phases of this project, Dr Davis Nwakanma and his team particularly Miss Cheryl Eniyou, for overseeing the molecular biology aspects of the study and for doing the quantitative PCR analysis; Dr Katie Flanagan and her team (especially Mrs Fatou Noho and Ms Jankey Jagne) for their training and support in whole blood culture and luminex assay, and for conducting the bioplex assay; and Dr Memuna Mendy who provided me training in neopterin analysis. To the laboratory staff of the MRC Keneba Field station, I am immensely grateful. Particular mention must be made of Karamo Camara, Musa Colley, Mustapha Bittaye, and Ebrima Danso. I am greatly indebted to my project staff especially my field supervisor Kabiru Ceesay, project nurses – Edrisa Sinjanka, Bakary Sarr, Kalilu Sanneh, Mohammed Sidibe, and all my village assistants; the staff at the MRC Keneba data office particularly Anup Muhammad Jahidul Hasan and Musa Jarjou; and the MRC Keneba drivers. To my friends Dr Patrick Nshe, Dr Julie Walabyeki, Dr Morenike Ukpong and Mrs Amie Jaiteh, and my maids Jara Sise and Fatou Sanyang, I am very grateful for standing by me all through this difficult period. Finally, I am most grateful to the

study participants and their parents without who there would have been no data.

2 Table of contents

3 List of tables

4 List of figures

5 List of models

6 Abstract

6.1 Background

The relative importance of malaria anaemia as a cause of childhood morbidity and mortality varies between and within regions. However, malaria anaemia remains an important cause of childhood morbidity and mortality. It has been estimated that globally, severe malaria anaemia occurs 1.42 to 5.66 million times per annum and kills an estimated 190,000 to 974,000 under-5 children. Studies from different countries endemic for malaria have emphasised the importance of anaemia in malaria-associated morbidity and mortality. Most of these studies have conclusively shown that severe malaria anaemia increases the risk of death in children with malaria; and in many reports, children with severe malaria anaemia often die before blood transfusion could be commenced. In addition, blood transfusion, which is the standard management for severe malaria anaemia, apart from not being available in many rural clinics, exposes the child to transfusion related infections such as human immunodeficiency virus (HIV). Better understanding of the pathogenesis of malaria anaemia therefore will enhance its prevention and management.

The pathogenesis of malaria anaemia is multifactorial and involves such mechanisms as immune and non-immune mediated haemolysis of parasitized and non-parasitized erythrocytes, bone marrow dysfunction, altered cytokine balance, nutritional deficits and interactions with common haemoglobinopathies and red cell defects such as glucose-6-phosphate dehydrogenase (G6PD) deficiency. An important component of the pathogenesis of malaria anaemia is iron delocalisation characterised by the sequestration of iron by the reticulo-endothelial tissues (the monocyte-macrophage system) as a result of malaria-induced inflammation. Iron sequestration creates a state of false iron deficiency which recovers after the inflammation has subsided. Therefore if the malaria-induced inflammation can be resolved more quickly, the degree and duration of malaria anaemia will be reduced. In addition, since the destruction of non-parasitized erythrocytes accounts for more than 90% of erythrocyte loss, use of anti-inflammatory drugs could minimize red cell loss.

Chloroquine is an antimalarial with proven anti-inflammatory properties. In addition, it is cheap, safe and has been shown to reduce iron delocalisation *in*

vitro. A proof of concept study was designed to investigate its potential use in the management of children with mild malaria anaemia.

6.2 Aims and hypothesis

The goal of the study was to investigate the effect of acute and continuing administration of chloroquine on haemopoietic response after a malaria episode. My hypothesis was that the anti-inflammatory and anti-macrophageal iron-loading effects of chloroquine will enhance erythropoietic recovery after a malaria episode.

6.3 Methodology

The study was designed as a randomised placebo controlled trial and was conducted over two malaria seasons. In the first year, the study consisted of four arms with a 2x2 design and only two arms in the second year. In the first year, the participants were initially randomised to receive antimalarial treatment with either chloroquine-sulphadoxine-pyrimethamine or co-artemether. All children with negative peripheral smear for malaria parasite by day three were subsequently randomised to receive either weekly chloroquine or weekly placebo until day 90. In the second year of the study, all the children were initially treated with co-artemether; subsequently, those with negative peripheral smear for malaria parasites were randomised to weekly chloroquine or weekly placebo as in the first year. Children randomised to weekly chloroquine and weekly placebo were followed up for three months. Various clinical and laboratory measurements were conducted on days 0, 3, 7, 15, 30, 45, 70 and 90. In year two of the study, no data were collected on days seven and 70. The main outcome measure was change in haemoglobin from day three to day 30 and from day three to day 90. Other outcome measures were

1. Changes in Hb in the placebo arms of the CQ-SP and ACT treatment groups
2. Changes in measures of inflammation – neopterin and cytokines
3. Changes in markers of iron status
4. Prevalence of sub-microscopic parasitaemia

6.4 Results

In 2007, 1445 children were placed under malaria surveillance, of which 105 malaria cases were recorded and 61 completed the 90 days follow-up. In 2008, of 1220 children under surveillance, 49 malaria cases were recorded, and 31 completed 90 days follow-up. There was no difference in Hb change from day three to day 30 and from day three to day 90 between the weekly chloroquine and weekly placebo arms. Although not statistically significant, the Hb change in children treated CQ-SP in 2007 was nearly twice the change in children treated with ACT at both days 30 and 90. The changes in the markers of iron status – MCV, MCH and ZnPP did not differ by treatment group and by randomisation group. During the acute malaria phase, neopterin concentration was high but by day 15, the levels had fallen to near zero levels and remained at this low level until day 30. Prevalence of sub-microscopic parasitaemia in the group was 15.1% and was similar in both randomisation arms. Iron deficiency was highly prevalent among the study participants. The independent predictors of Hb change were Hb at day 0, presence of iron deficiency, age of the child and height-for-age z score.

6.5 Conclusions

Giving weekly chloroquine at a dose of 5mg/kg to children with mild anaemia associated with malaria did not confer any advantage to bone marrow recovery compared to children who received placebo. The data, however, suggests that the initial therapeutic dose of chloroquine (10mg/kg/day over three days) could have some positive effects on bone marrow recovery post malaria. The Hb recovery following treatment for malaria is determined by the age of the child, the Hb at diagnosis, the presence or absence of iron deficiency, and the height-for-age z score.

7 Author's declaration

I declare that all the work within this thesis, except where otherwise acknowledged, is my work. However, executing the research was a team work. I am employed as a full time Research Clinician with the MRC International Nutrition Group, and based at the Keneba Field Station in The Gambia. During various stages of the project I received technical support from various members of the Group especially from Dr Sharon Cox who was my main adviser on the basic science aspect of the project. I also received technical support from my supervisors Dr Conor Doherty, Professor Lawrence Weaver and Professor Andrew Prentice. However, I was responsible for the overall design of the project, the design of the various study instruments, and general project supervision. The data were collected by my field team under my supervision, and entered into an access database by the data entry clerks of the MRC Keneba Field Station. I was responsible for all the laboratory procedures except malaria microscopy, quantitative PCR, and Luminex assay. Where I was not primarily responsible for a particular laboratory procedure whether due to technical or logistic reasons, I received relevant training in those aspects and assisted as much as possible. I carried out all of the data analysis and interpretation described in this thesis with advice from Dr Anthony Fulford, a statistician with the MRC International Nutrition Group, and Dr David Young, a statistician with the Strathclyde University, Glasgow. I was responsible for all the writing of the thesis. Where existing texts have been adapted e.g. texts from manuals of reagents, these have been appropriately acknowledged.

8 Abbreviations used

95%CI	95% Confidence Interval
aCD28	anti-CD28
aCD3	anti-CD3
ACT	Artemisinin-based combination therapy
ACTH	adrenocorticotropic hormone
BFU-E	burst-forming unit erythron
BMI	body mass index
C3	Complement component 3
CD163	cluster of differentiation 163
CD4	cluster of differentiation 4
CDC	Centers for Disease Control
CFU-E	colony forming unit erythron
CFU-e	colony-forming unit erythron
CP	ceruloplasmin
CQ	chloroquine
CQ-SP	chloroquine-sulphadoxine-pyrimethamine
Cr	chromium

C_T	threshold cycle
DAT	direct antiglobulin test
DDT	dichlorodiphenyltrichloroethane
df	degree of freedom
DISC	death-inducing signalling complex
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
DSS	Demographic Surveillance Systems
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
ETF	early treatment failure
FFQ	food frequency questionnaire
FI	florescence intensity
FPN1	Ferroportin1
FU	follow-up
FW	field worker
G6PD	glucose-6-phosphate dehydrogenase
GDP	gross domestic productivity
GTPase	guanosine triphosphatase

HAZ	height-for-age Z score
Hb	haemoglobin
HFE	human haemochromatosis protein
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IFN-α	interferon-alpha
IFN-γ	interferon-gamma
IL-1	interleukin-1
IL-10	interleukin-10
IL-12	interleukin-12
IL-13	interleukin-13
IL-17	interleukin-17
IL-18	interleukin-18
IL-4	interleukin-4
IL-6	interleukin-6
IMCI	integrated management of childhood illnesses
IRE	iron-responsive element
IRP	iron regulatory proteins
K_2HO_4	potassium phosphate
LEAP-1	liver-expressed antimicrobial protein 1

Log_{10}	log to base 10
Log_e	natural logarithm
LPS	lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MCH	mean corpuscular haemoglobin
MCV	mean corpuscular volume
MIF	macrophage migration inhibitory factor
MOMP	mitochondrial outer membrane permeabilization
MP	malaria parasite
MRC	Medical Research Council Laboratories, The Gambia
mRNA	messenger ribonucleic acid
Na_2PO_4	sodium phosphate
NCHS	National Centre for Health Statistics
ng/ml	nanogram per millilitre
NO	nitric oxide
NRAMP2	natural resistance associated macrophage protein 2
OR	odds ratio
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PCV	packed cell volume

PfSE	*Plasmodium falciparum* schizont extract
PHA	phytohaemagglutinin
PHC	primary health care
PI	principal investigator
PPD	purified protein derivative
RBC	red blood cells
RHT	regional health team
RN	resident nurse
ROS	reactive oxygen species
SD	standard deviation
SDQ	socio-demographic questionnaire
SEM	standard error of mean
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1
SME	second malaria episode
TB	tuberculosis
TBA	Traditional Birth Attendants
TfR	transferrin receptor
Th1	T helper 1
TNFR	tumour necrosis factor receptor
TNFRSF6	tumour necrosis factor receptor superfamily, member 6

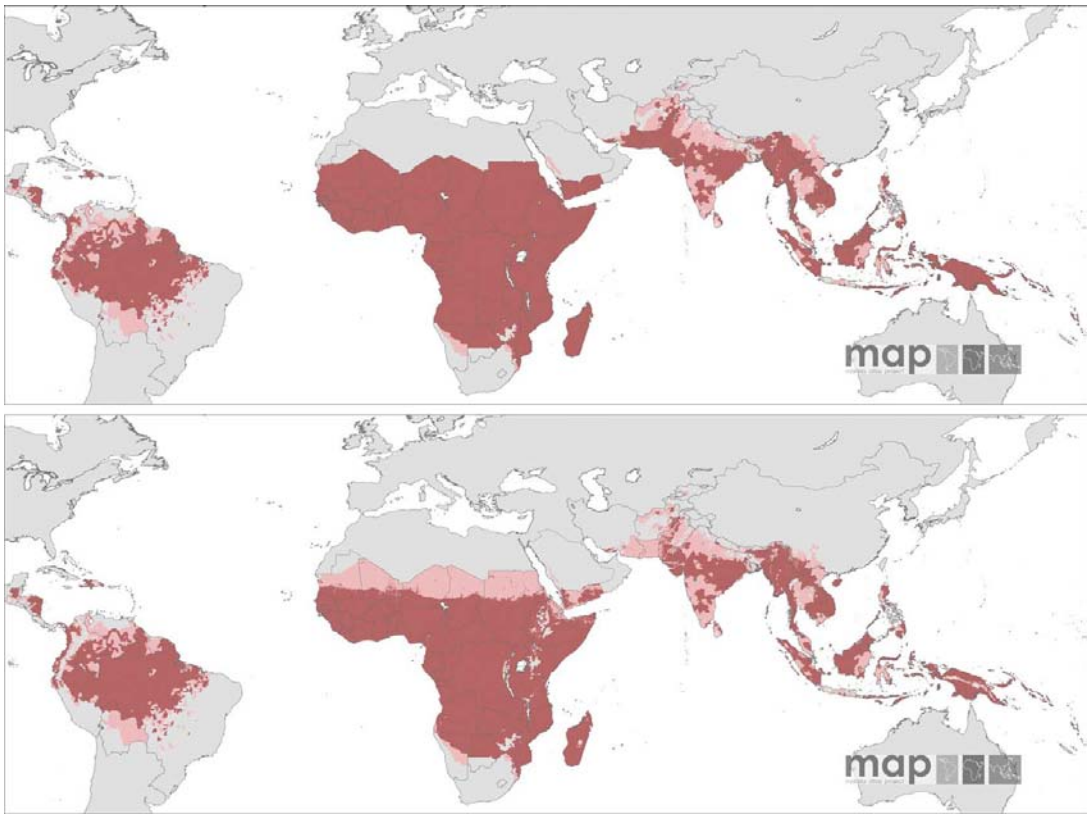
TNF-α	tumour necrosis factor alpha
TT	tetanus toxoid
uRBC	uninfected red blood cells
US	United States of America
USF2	upstream stimulatory factor 2
UTI	urinary tract infections
UTR	untranslated region
VA	village assistant
VHW	village health workers
WAZ	weight-for-age Z score
WHO	World Health Organisation
WHZ	weight-for-height Z score
ZnPP	zinc protoporphyrin

1 Chapter 1 – Background

1.1 Global burden of malaria

The 2009 state of the world's children indicates that under-five mortality rate in much of the developing world is still unacceptably high[1]. Nearly all of these countries with the highest mortality lie within the malaria belt[2-3] (Figures 1, 2 & 3); and directly or indirectly, malaria contributes significantly to under-five mortality, especially in sub-Saharan Africa[4-7]. About 247 million (189 million to 327 million) clinical cases of malaria occurred globally in 2006 resulting in 881 000 (520 000 to 1,212,000) deaths, 85% of which were in children below five years of age[3].

It has long been known that there is a strong correlation between malaria and poverty, with some reports suggesting that malaria might actually be a cause of poverty[8-10]. It is estimated that malaria causes a decrease in economic growth in highly endemic countries by more than one percentage point per year[11]. Gallup and Sachs[8] compared economic growth from 1965 to 1995 between malaria endemic countries and non-malaria endemic countries. They showed that after controlling for other possible causes of slow economic growth, countries with a significant malaria burden in 1965, had 1.3% less economic growth per year when compared with countries without malaria. However, in countries with reductions in malaria over 1965 – 1990, there was an associated 0.3% rise in annual economic growth for a 10% reduction in the malaria index. In real terms, it is estimated that malaria results in a loss of more than US$12 billion a year in gross domestic productivity (GDP)[12], with Africa losing more than US$2000 million per annum to malaria[13-14]. Onwujekwe et al[15] in Nigeria, estimated that the cost of treating malaria illness accounted for 49.9% of curative health care costs incurred by households, with an average malaria expenditure of $1.84 per household per month, and $2.60 per month when treated along with other diseases. These findings suggest that malaria greatly facilitates the poverty-infection cycle in endemic countries; it impacts negatively on the family income and, by extension, the quality of life of both the child and the entire family.



Areas were defined as stable (dark-red areas, where PfAPI ≥0.1 per thousand pa), unstable (pink areas, where PfAPI <0.1 per thousand pa), or no risk (light grey). Dark grey – no data available

Figure 1: *P. falciparum* malaria risk defined by annual parasite incidence (top), temperature, and aridity (bottom) (From Guerra et al, PLoS Med. 2008 February; 5(2): e38. doi: 10.1371/journal.pmed.0050038)

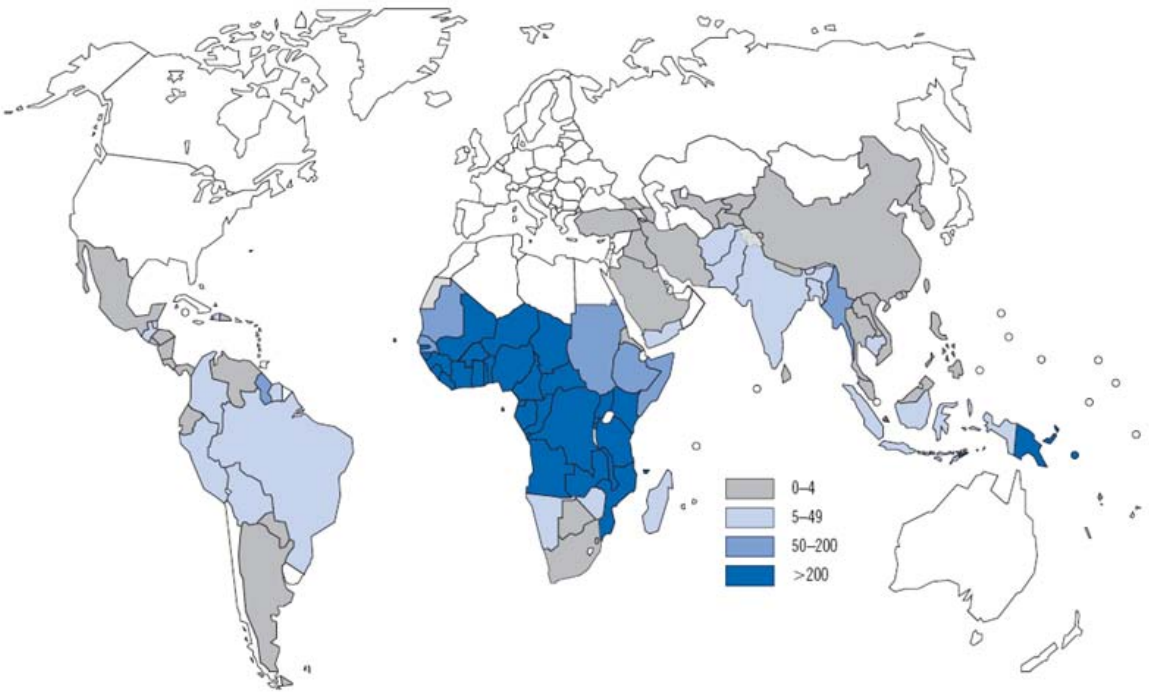


Figure 2: Estimated incidence of malaria per 1000 population, 2006 (http://www.rbm.who.int/wmr2005/html/map1.htm), accessed 09/03/09 (World Malaria Report 2008)

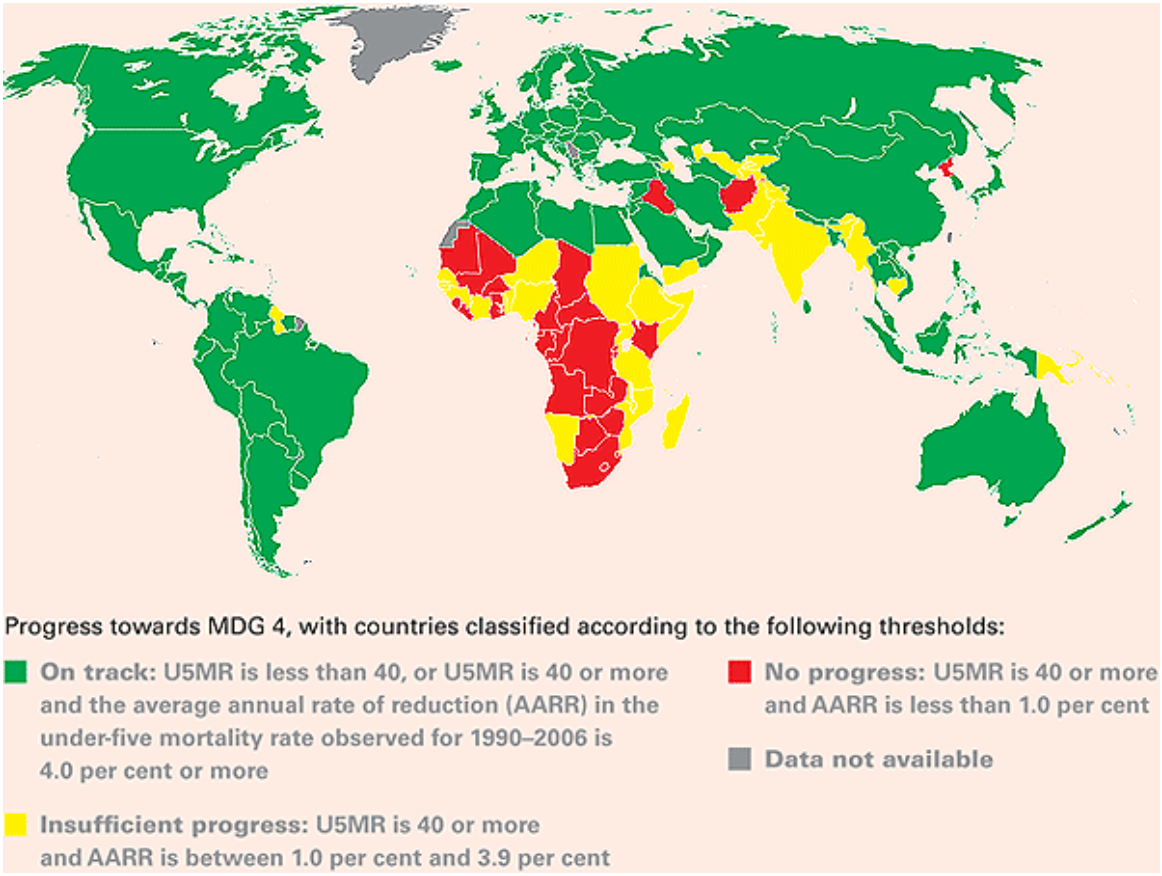


Figure 3: Under-five mortality rate, 2007 (http://www.unicef.org/progressforchildren/2007n6/index_41802.htm, accessed 28/06/2010)

Recent reports have explored the anthropologic perspectives of malaria by looking at the social burden of malaria. Sachs and Malaney[9] described the social burden of malaria as those costs that result from changes in household behaviour in response to malaria. Such costs include decisions related to reproduction, education and economic matters, which have long term impact on economic growth and development. They proposed that the high malaria associated child mortality influence fertility decisions of households[9]. The child survivor hypothesis predicts "that a high burden of malaria will lead to a disproportionately high fertility rate and an overall high population growth rate in regions of intense malaria transmission"; the consequence of which will be reduced investments in education per child, and increased human capital costs for women[9]. Jones and Williams[16] took this further by considering other factors that contribute to the social burden of malaria such as influences of culture, beliefs and political contexts, all of which affect perceptions, individual behaviour, social structure, and social action[16]. They argued that because of the high prevalence of malaria in endemic areas, uncomplicated malaria has become socially acceptable leading to a low social pressure to seek treatment, provide

money for treatment to close relatives, and to comply with completing malaria treatment. Such social perceptions of the 'sick role' in uncomplicated malaria could further worsen the gender inequity in most malaria endemic societies through differences in definition of appropriate 'sick roles' for both men (the traditional bread winners) and women (the traditional subordinates). While men could seek prompt treatment without any need for consultation, the women are usually expected to continue their primary roles as caregivers and to consult other household members before seeking treatment[16]. On the other hand, severe forms of malaria are likely to be considered a result of supernatural influences rather than being related to malaria. All of these have severe social consequences, which affect the sick individual, his or her family, and the community as a whole[16]. Malaria, therefore, remains an important global public health problem, requiring urgent interventions to mitigate its impact.

1.2 Malaria anaemia in children

Anaemia is one of the three most important life threatening complications of malaria; the other two being cerebral malaria and respiratory distress[17]. In many malaria endemic areas, anaemia is a very important public health problem among children[18]. In 2007, there were at least 244 million anaemia among children aged zero to five years worldwide [19], with prevalence varying from region to region. In Asia, the prevalence of anaemia among this age group was 39.6% in 2007 with a total of 139 million children affected; in south and central America and Caribbean where about 19 million children aged zero to five years were anaemic in 2007, the prevalence was 35.6%, while Africa had a prevalence of 58.7% with about 87.5 million children aged zero to five years anaemic. Within Africa, the prevalence varies from 33.9% in North Africa to 66.7% in East Africa (Table 1). The prevalence of anaemia in children aged zero to five years in West Africa was about 61.9% in 2007 with about 29 million children affected.

Anaemia as a common cause of morbidity and mortality in The Gambia has been recognised since the 1960s[20-21]. A 1991 nationwide survey on the prevalence of Vitamin A and Iron deficiency in women and children in The Gambia found that among a nationally representative sample of children aged one to five years, 76% had a haemoglobin (Hb) less than 11g/dl while 15% had Hb levels less than 7g/dl[22]. There are suggestions that most of these are due to malaria[23-26]. However, many factors contribute to anaemia in malaria-endemic countries like

the Gambia including micronutrient deficiencies particularly iron, folate, vitamin B12, ascorbic acid and vitamin A deficiencies [19, 27-28]. Age is also an important determinant of anaemia in malaria endemic countries. Younger children are at a higher risk of malaria anaemia than older children [29-31]. Among a cohort of 338 children aged six to 40 months in a malaria holoendemic area of Tanzania, Premji et al [29] found that the prevalence of anaemia decreased with increasing age while the severity of anaemia decreased with increasing age. Similar findings have been reported from Kenya[30] and Mozambique [31].

Table 1: Estimated prevalence of anaemia in children 0-5 years old, 2000-2007 (Source: 6th report on world nutrition situation)

Sub-region	Prevalence (%)			Number (thousand)	Rate (percentage points per year)
	2000	2005	2007	2007	2000-2007
East Africa	70.7	68.8	66.7	34 524	-0.6
Central Africa	70.3	64.9	62.7	13 604	-1.1
North Africa	42.2	36.3	33.9	7 467	-1.2
Southern Africa	47.5	42.3	40.7	2 485	-1
West Africa	69.3	64.2	61.9	29 387	-1.1
Region	64.6	60.8	58.7	87 467	-0.8

Calculating the precise contribution of malaria to anaemia is extremely difficult primarily because, as already noted above, many of these children also suffer from other conditions which could cause anaemia[32]; and also because of differences in methods of Hb estimation and diagnostic accuracy[33]. Using a variety of sources, including original scientific data, Murphy and Breman[33] calculated that every year, severe malaria anaemia occurs 1.42 to 5.66 million times, and kills an estimated 190,000 to 974,000 children below five years of age. In an area of Tanzania where malaria transmission is intense and perennial, malaria accounted for 60% of severe anaemia among infants[34].

The relative importance of malaria associated anaemia compared to the other complications of malaria varies across geographical locations and seasonality of malaria transmission. For example, in Zambia, severe malaria anaemia accounted for 10% of all paediatric admissions and a case fatality rate of about

8%[35]; while in The Gambia, over a two year period, severe malaria anaemia accounted for 17% of all the malaria admissions or 7% of all the paediatric admissions[36]. In Ghana, a study found a severe anaemia prevalence of 22.1% across a high transmission season, and 1.4% at the end of a low transmission season[37]. In two hospitals in Malawi located in areas with different seasonal patterns of malaria transmission, the contribution of malaria-associated severe anaemia to malaria-related morbidity and mortality was respectively 8.5% and 54% in the area with sustained year round transmission and 5.2% and 32% in the area with fluctuating pattern of infection[38].

Severe anaemia increases the risk of death in children with malaria[39]. In The Gambia, Bojang and co-workers[36] reported that as many as 57% of deaths due to malaria anaemia occurred within four hours of admission, most occurring before blood transfusion could be commenced. And in Zambia, three out of six deaths in one arm of a randomised controlled trial were directly related to malaria; the three malaria associated deaths had initial packed cell volumes (PCV) of 14%, 12% and 19%, and all died within the first 48 hours of admission[40]. In addition, blood transfusion which is the standard management for severe malaria anaemia exposes the child to the risk of transfusion related infections such as Human Immunodeficiency Virus (HIV) and hepatitis[41], and is usually not available in rural clinics. Better understanding of the pathogenesis of malaria, will enhance prevention and management of malaria anaemia in children.

2 Chapter 2 – Iron Metabolism

Iron, a major constituent of haem, and closely involved in oxygen transport in the body, is an essential component of many metabolic processes in all living things. An adult human being contains 2-6 grams of iron, two thirds of which is found in haemoglobin (Hb), and the rest stored in bone marrow, liver, spleen and muscles. About 15% of the body's iron is found in various enzymes and myoglobin, and another 15% stored as ferritin (the major iron storage protein in the body); only about 1% is carried in the plasma, usually by transferrin, the major iron transport protein in the body (Figure 4).

The regulation of body iron depends on iron absorption from the intestines; there are no known excretory pathways for iron in the body. Only about 1-2mg of the iron consumed in food is absorbed, depending on the physiological need for iron, dietary iron intake, bioavailability of dietary iron and the ability of the mucosal cell to adjust iron absorption to physiological demands and to the amount and bioavailability of dietary iron (adaptation) [42]. Much of the body's daily need of 20-24 mg of iron is provided by macrophages through catabolism of haemoglobin from senescent erythrocytes. A variable amount of iron is lost daily from the sloughing of the skin and mucosal cells.

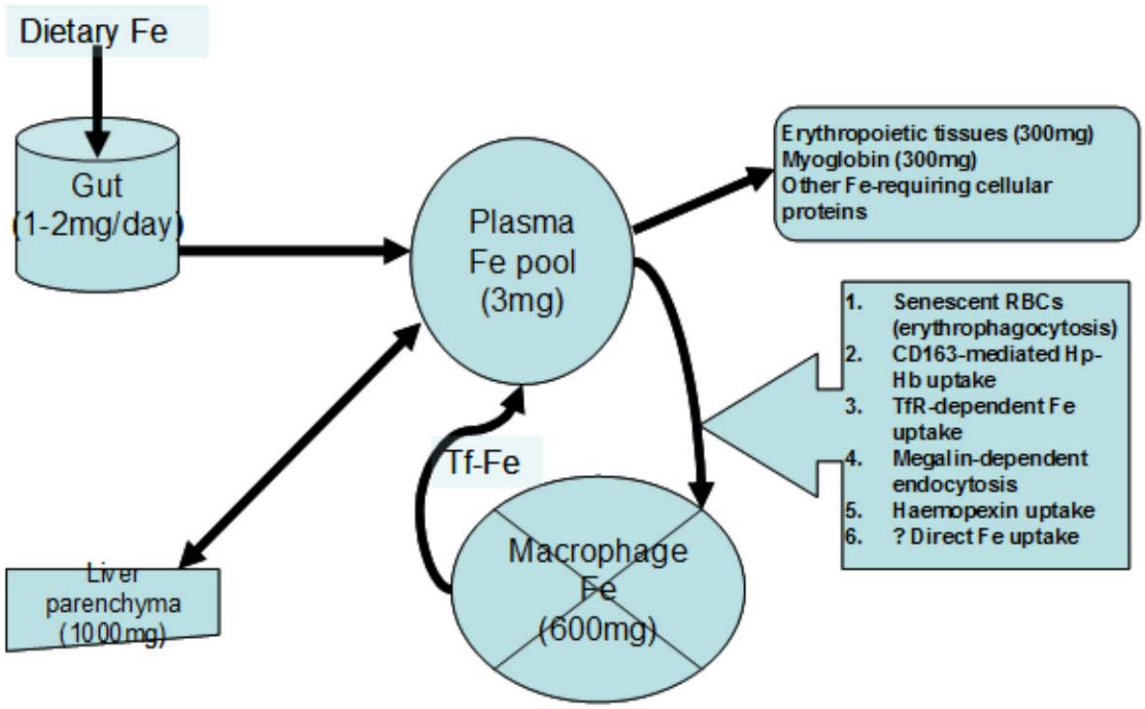


Figure 4: Normal iron metabolism (Iron values adapted from Andrews NC. N Engl J Med 1999;341:1986–95)

The duodenum is the main site of iron absorption. Ingested iron is reduced from the ferric to ferrous form by a cytochrome-b-like ferrireductase at the intestinal brush border, and transported actively across the apical enterocyte membrane by divalent metal transporter 1 (DMT1), also called Natural Resistance Associated Macrophage Protein 2 (Nramp2) (Figure 5). Some of the iron in the enterocyte is exported into the plasma across the basolateral membrane by the action of ferroportin 1 (an active iron exporter), while the rest remain as ferritin. The iron remaining as ferritin is lost when the enterocyte sloughs off at the end of its life cycle. The iron released into the plasma is usually in the ferrous form, and will need to be oxidized to the ferric form by hephaestin (a ferrooxidase) before it can be bound to transferrin, the main iron transport protein, and with which iron is carried to various sites for storage or for utilization in the various body's metabolic processes.

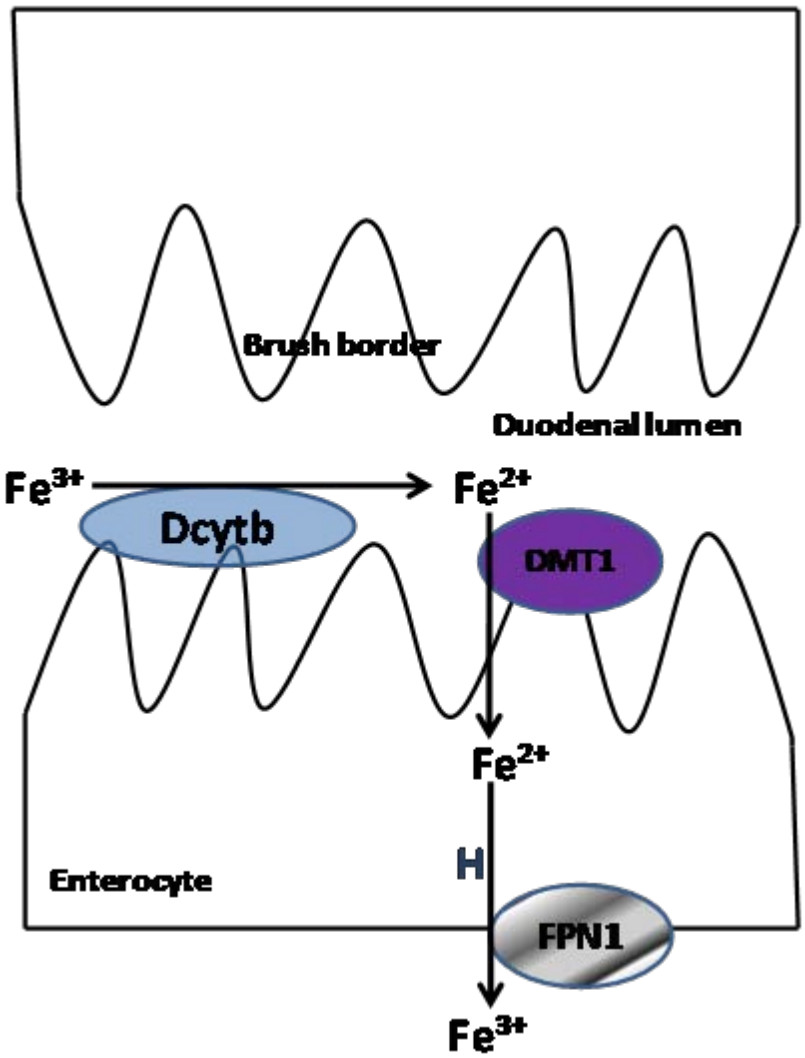


Figure 5: Duodenal iron transport. Dcytb = duodenal cytochrome B; DMT1 = divalent metal transporter 1 (Nramp2); FPN1 = ferroportin 1; H = hephaestin; Fe^{3+} = ferric iron; Fe^{2+} = ferrous iron

2.1 Transferrin-dependent iron uptake mechanisms

Several transferrin-dependent iron uptake mechanisms help body cells to extract iron from the iron-transferrin complex. One of such mechanism involves the use of transferrin receptor1 (TfR1). Saturated transferrin binds with TfR1 to form transferrin-TfR1 complex, causing a cavity to form on the cell membrane. Closure of the cavity produces an endocytic vesicle which is hydrolysed by the action of a proton pump to release the iron from the transferrin-TfR1 complex. The released ferric iron is then reduced to ferrous iron by Steap3 (six-transmembrane epithelial antigen of the prostate 3)[43-44], allowing DMT1 to transport the iron from the endosome into the cytoplasm. The iron depleted transferrin-TfR1 complex is acted on by the neutral pH of the plasma to liberate the apotransferrin (iron depleted transferrin).

Transferrin receptor 2 (TfR2) on the other hand is found only in the hepatocytes, duodenal crypt cells and the erythroid precursors. They are thought to influence hepcidin expression by sensing transferrin bound iron. Hepcidin is a 25 amino acid hormone produced by the hepatocytes. It modulates the ferroportin-mediated release of iron by binding directly to the ferroportin causing it to be carried into the cells where it is degraded. Since ferroportin is responsible for exporting iron out of the cells into the plasma, reduction in the concentration of ferroportin will reduce the amount of iron transferred to the plasma from the cells, and increase the amount of iron stored.

A third mechanism of transferrin dependent iron uptake involves megalin-dependent cubilin-mediated endocytotic process. Megalin is a 600kDa transmembrane protein belonging to the LDL-receptor family. Cubilin on the other hand is a 460kDa peripheral membrane protein and is identical to the intestinal intrinsic factor-vitamin B_{12} receptor (for excellent reviews of the physiology of cubilin and megalin, see[45-47]). While megalin is expressed in many epithelial cells, cubilin is expressed on the apical pole of absorptive epithelia including the renal proximal convoluted tubule, visceral yolk sac, ileum and placenta[45, 47]. Cubilin lacks transmembrane domain and no known signal for endocytosis[46]. It therefore requires megalin for its internalization. The two receptors bind tightly to each other and co-localize in several tissues; and they are both important for normal renal tubular reabsorption of proteins[46-47]. With respect to iron metabolism, megalin-cubilin receptor mechanism mediates the

reabsorption of transferrin and haemoglobin[48-49], a process that is thought to be harmful to the kidney under pathological conditions with increased glomerular filtration rate[47]. Animal studies showed lack of vesicular accumulation of transferrin associated with high urinary excretion of transferrin in cubilin deficient dogs and megalin deficient mice compared to normal control animals[48]. Also megalin knockout mice lacked vesicular accumulation of haemoglobin unlike normal mice[49].

2.2 Macrophageal iron flux

Macrophages are responsible for the removal of senescent red blood cells from the body. Senescent red blood cells are phagocytosed by reticuloendothelial macrophages, within which the erythrocyte lyses to release haemoglobin. The haemoglobin is degraded by haem oxygenase, which is localised in the endoplasmic reticulum, to release biliverdin, carbon monoxide and ferrous iron[50]. Part of the iron is stored in the macrophage while the rest is exported to the plasma by ferroportin. This macrophageal recycling accounts for most of the 20-24mg of iron required daily for haemoglobin production (Figure 4). Impairment of the macrophageal iron release mechanism will result in reduced serum iron.

Macrophages acquire iron through several mechanisms: erythrophagocytosis of senescent erythrocytes; transferrin-dependent iron uptake mechanisms, megalin-dependent cubilin-mediated endocytotic process[45-47] and possibly direct uptake of free iron from the plasma. The iron released from the catabolism of senescent erythrocytes appears to be the largest source of iron in the reticuloendothelial system (Figure 4). Macrophages also acquire iron in the form of haemoglobin bound to haptoglobin. It has been estimated that as much as 20% of normal erythrocyte destruction occurs intravascularly releasing haemoglobin into the circulatory system. Since haemoglobin is a powerful pro-oxidant, it is rapidly removed from the circulation by haptoglobin, an acute phase protein. The haemoglobin-haptoglobin complex is cleared by the haemoglobin scavenger receptor – CD163, expressed exclusively in monocytes and macrophages[51]. The CD163 mediates the endocytosis of the haemoglobin-haptoglobin complex and its subsequent degradation[51]. There are suggestions that the reticuloendothelial system is also able to acquire iron from haemopexin, a plasma glycoprotein which binds circulating free haem in the plasma and are cleared by haemopexin

receptors. Some workers have reported detection of these haemopexin receptors on human monocytic cell lines[52-53], but their importance in the macrophageal iron uptake is not clear[54].

Macrophageal iron recycling is an important source of iron supply for erythropoiesis. Most of this iron comes from erythrophagocytosis. Iron is released from the macrophages into the plasma in a biphasic fashion – an early phase and a late phase[55-60]. Macrophages release iron either in the form of low molecular weight iron[57, 61-62], or as Hb, haem and ferritin[55-60, 63]. In the early phase of macrophageal iron release, about two-thirds of Hb-derived iron is returned to the plasma within the first few hours predominantly as Hb; while in the late phase the rest of the Hb-derived iron is released slowly over days and weeks [55-60] chiefly as ferritin and low molecular weight iron[60].

2.3 Nitric oxide and macrophageal iron flux

The messenger ribonucleic acids (mRNA) that transcribe for most of the molecules involved in iron metabolism contain iron responsive elements (IRE) in the 5´un-translated regions (UTR), including transferrin receptor and ferritin. Binding of these IREs to Iron regulatory proteins (IRP), of which there are two forms (IRP 1 and IRP 2), downregulates the translation of these molecules, and thus modulate iron uptake and storage. Increase in plasma iron causes a failure of the IRPs to bind to IREs, leading to increased translation of ferritin and degradation of transferrin receptor mRNA. The end result is increased iron storage. Conversely, reduced plasma iron will stimulate the binding of IRPs to IREs leading to reduced degradation of transferrin receptor mRNA and decreased translation of ferritin, leading to increased acquisition of iron by transferrin.

There is consensus that nitric oxide (NO) induces the binding of IRP to IRE[64-69]; however, there is less agreement on how this action affects iron metabolism. While some studies have reported NO-induced increase in ferritin synthesis by J774 stimulated cells (stimulated murine macrophage cell line) [66], others reported a decrease in ferritin synthesis[65]. Similarly, some reports suggest that IRP1 stimulation by NO causes increase in TfR mRNA levels[68-70], contrary to the findings of other workers[71-72].

These conflicting findings may be explained by the observation that IRP1 and IRP2 can be stimulated to exert opposing effects by the same immunological

stimuli[67]. Although NO release by activated macrophages up-regulates IRP1 activity, its overall effect is decreased iron uptake by macrophages[64]; probably due to a direct effect of NO on cellular iron availability in addition to the effect of NO on IRP1. Also, NO has different biological effects depending on its redox state, particularly the two redox-related species ($NO^{\bullet}$ and NO^{+})[73]. Thus while $NO^{\bullet}$ increased IRP1-IRE binding, and therefore increased TfR mRNA, NO^{+} prevented the binding of IRP1 to IRE leading to degradation of TfR mRNA (for review, see Richardson et al.[73]). Although inconclusive, it would appear that currently available evidence supports a dual role for NO in reduced iron availability during inflammation – counterbalancing the effect of pro-inflammatory cytokines and causing reduced cellular iron uptake. The molecular interactions of the IRE/IRP regulatory network are detailed in a recent review[74].

3 Chapter 3 – Pathogenesis of Malaria Anaemia

3.1 Introduction

The pathogenesis of malaria anaemia is multifactorial[32]. The mechanisms involved include immune and non-immune mediated haemolysis of parasitized and non-parasitized erythrocytes, bone marrow dysfunction, and altered cytokine balance. Other mechanisms are nutritional deficits and interactions with common haemoglobinopathies and red cell defects such as glucose-6-phosphate dehydrogenase (G6PD) deficiency (Figure 6).

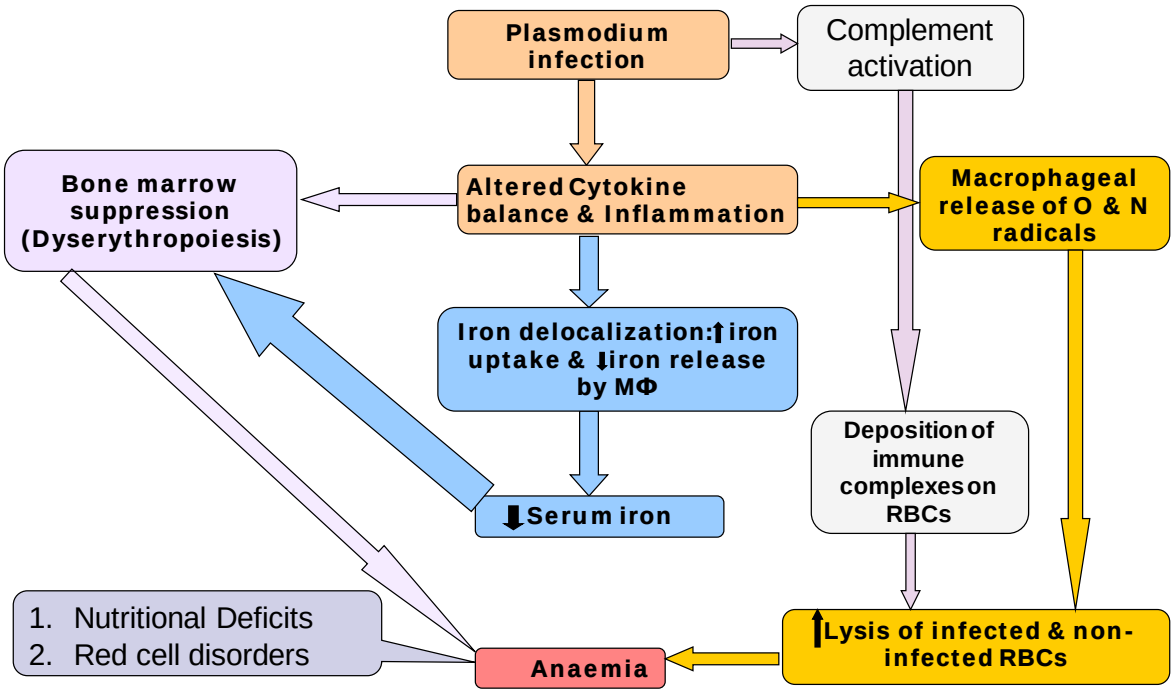


Figure 6: Pathogenesis of Malaria Anaemia (adapted from Nweneka, et al. Trans R Soc Trop Med Hyg. 2010 Mar;104(3):175-84. Epub 2009 Sep 23). MΦ = macrophages; O = oxygen; N = nitrogen; RBCs = red blood cells

3.2 Non-immune and non-specific immune-mediated clearance of erythrocytes in malaria infection

Millions of red cells are destroyed at the end of each asexual reproductive cycle of the *plasmodium* organism when they rupture to release merozoites[75]. *Plasmodium*-induced changes in erythrocyte membranes and splenic function cause increased haemolysis and clearance of parasitized erythrocytes through specific and non-specific immune-mediated mechanisms. Splenomegaly was associated with enhanced clearance of ^{51}Cr-labelled autologous erythrocytes in 25 adult patients with acute *P. falciparum* malaria and a lower mean PCV

compared with normal controls or patients with acute malaria without splenomegaly[76]. In another report, these authors found increased erythrocyte destruction with clearance of *P. falciparum* or *P. vivax* parasites independent of complement or antibody mediation. They suggested a mechanism of non-specific activation of the reticuloendothelial function associated with the parasitic infection[77-78].

Schwartz and others[79] examined human red cells experimentally infected with *P. falciparum* and noticed that parasite development was accompanied by distinct changes in the proportion of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine in the transbilayer of the red cell membrane compared to uninfected cells from the same population. There were increased amounts of phosphatidylethanolamine and phosphatidylserine and a decreased amount of phosphatidylcholine in the exosplasmic leaflet of infected red cells; thus altering the normal asymmetry of the red cell membrane phospholipids. Such disorganisation, the authors suggest, could result in changes in cell membrane permeability and susceptibility to early haemolysis[79]. Other workers have also reported alterations in the structural contents of membranes of both infected and uninfected erythrocytes[80-81]. *In vitro* and *in vivo* studies by Sabolovic and colleagues[82] also found alterations in the membrane characteristics of uninfected red cells in *plasmodium* infection. They reported that the presence of *plasmodium* infection reduced the net negative charge of the uninfected cells and at the same time increased their resistance to linoleic acid induced lysis, a process that began 24 hours after infection, peaked on the day 3 and returned to normal after massive appearance of parasites in the blood[82]. Such reductions in RBC net negative charges have been shown to increase the chances of the red cells being trapped in organs and subsequently destroyed[83]. Reduction of the negative charges of uninfected erythrocytes also causes increased adsorption of fatty acids; predisposing the cells to a higher susceptibility to free radical attack causing haemolysis [82]. Erythrocyte deformability at admission in 42 Thai patients with severe *falciparum* malaria was significantly reduced compared with normal healthy controls, and was significantly correlated with the degree of anaemia[84] and with the severity of illness[85].

In a model comparing the relative contributions of dyserythropoiesis, haemolysis, and clearance of parasitized and unparasitised erythrocytes during acute malaria in non-immune patients, Jakeman et al[86] showed that the destruction of unparasitised erythrocytes was the most important determinant of Hb level. They argued that since (according to their model) dyserythropoiesis played an insignificant role in malarial anaemia, and the anaemia during acute infection occurred before a substantial antibody response to either parasite or erythrocyte had been generated, the major determinant of malarial anaemia was the destruction of uninfected cells by phagocytosis. This is supported by observed changes in the membranes of unparasitised erythrocytes during malarial infection[87]. Data from other studies have also shown that haemolysis of non-parasitised erythrocytes accounts for more that 90% of erythrocyte loss during acute malaria[88-89].

Plasmodium infection is associated with complement activation[90]. Erythrophagocytosis and complement-mediated haemolysis are enhanced by the deposition of immune complexes on the cell membranes of infected and uninfected erythrocytes [89, 91-97]. *Plasmodium* infection activates both classical and alternative complement pathways [98-99]. The Classical complement pathway was activated in a dose dependent manner by *P. falciparum* infected erythrocytes incubated with complement and varying amounts of different types of immune sera; while the alternative complement pathway was activated both in the presence and absence of immune sera[99]. Stanley and co-workers[99] suggested that such interactions of complement components with infected erythrocytes, even if not producing immediate haemolysis, could alter critical membrane characteristics increasing the flow of ion and other metabolites across the membrane; while the C3b deposited on the red cell surface could facilitate opsonisation and destruction of the cells by monocytes and polymorphonuclear leukocytes. Earlier studies by Lee et al[100] had indeed shown that in patients with uncomplicated *P. falciparum* malaria, there is rapid clearance of IgG sensitized red cells by the spleen which persists during recovery.

Direct antiglobulin test (DAT, Direct Coombs test) detects complement proteins bound to the surface membranes of circulating erythrocytes, and can therefore be used as a marker of complement activation and complement-mediated

haemolysis. While some workers have argued that a positive DAT might not necessarily be associated with excessive haemolysis of non-parasitized erythrocytes [101-103], others have found a strong correlation between DAT positivity and malaria anaemia [92, 104-105]. In Ghana, DAT positivity was significantly associated with lower Hb levels[94, 96]. In 15 healthy volunteers experimentally infected with *Plasmodium falciparum*, significant complement activation in the sub-clinical and early clinical stages of malaria was demonstrated even before parasitaemia became clinically detectable[92]. Some reports have also demonstrated reduced life span of unparasitised red cells in the presence of *P. falciparum* infection[78]. Facer and co-workers[104] found a 50% incidence of Coombs positivity using monospecific antisera in a group of Gambian children with malaria, most of whom had erythrocytes sensitized with C3, but not to IgA or IgM[104]. They suggested that this might contribute to the pathogenesis of anaemia in *falciparum* malaria through a Type III complex-mediated hypersensitivity involving parasite antigen-antibody complexes[104]. Studies have also reported increased susceptibility of uninfected red cells to lysis and peroxidation of the membrane by activated monocytes[106]. Part of the mechanisms behind the increased complement mediated destruction of red cells might include the loss of complement regulatory proteins especially CR1 and CD55 seen in the red cells of children with severe malaria anaemia which compromises their ability to bind immune complexes and increase their susceptibility to complement-mediated destruction[95, 107-109].

3.3 Bone marrow dysfunction

The role of bone marrow dysfunction in the pathogenesis of malaria anaemia has been extensively investigated[110-113]. Wickramasinghe and Abdalla[114] have reviewed the blood and bone marrow changes occurring in acute and chronic malaria. In summary, acute uncomplicated malaria is associated with bone marrow changes spanning the entire spectrum of cellularity, being predominantly hypercellular in Gambian children[114]. There is also associated normoblastic erythropoiesis with gross deformation of the nuclei even in the presence of normal levels of serum folate and vitamin B_{12} and stainable bone marrow iron. There are increased numbers of macrophages which contain ingested malaria pigment, parasitized and non-parasitized erythrocytes as well as other blood cells including granulocytes, lymphocytes and plasma cells[114]. The formation of these cells during acute malaria was independent of either vitamin

B_{12} or folate deficiency or any impairment of the metabolic pathways of these vitamins[114]. In a group of Gambian children with chronic malaria, spontaneous remission of anaemia was noticed with disappearance of parasitaemia; and a consistent finding in these children was marked dyserythropoietic changes in their bone marrows[115]. In some reports these dyserythropoietic changes persists well into the convalescent periods[114]; however, there is no consensus on the pathogenetic mechanism of the dyserythropoiesis.

Some studies have suggested the existence of soluble mediators in splenic and bone marrow cells, probably macrophage migration inhibitory factor[116-117], that are capable of inhibiting the response of erythroid progenitor cells (colony forming unit-erythroid (CFU-e) and burst forming unit-erythroid (BFU-e)) to erythropoietin in the presence of *Plasmodium*[118-120]. Other studies[121] have not found similar abnormalities. Abdalla and colleagues[121] observed that although varying widely, there was no deficiency in erythroid progenitor cells in Gambian children with *falciparum* malaria, and that growth of colony forming unit erythroid (CFU-e) was observed even in the absence of added erythropoietin when autologous serum was used in the culture system. They concluded that children with *P. falciparum* malaria showed no major abnormality in their erythroid progenitor cells and that the perturbation of erythropoiesis in such children occurs mainly in the morphologically recognizable erythroid precursor cells. They further proposed that the wide variation observed in the number of CFU-e and BFU-e in different patients, and the correlations between the number of BFU-e and parasitaemia and the number of BFU-e and CFU-e are all probably largely related to the changing clinicopathological situation in patients with malaria and anaemia[121].

3.4 Erythropoietin (EPO) production during malaria

Reduced bone marrow stimulation by erythropoietin could explain the observed dyserythropoiesis in malaria. However, the effect of *plasmodium* infection on erythropoietin production is unclear. Both human and animal studies have found appropriate erythropoietin response to *plasmodium* infection[110, 122-130], suggesting that the inadequate erythropoiesis is unlikely due to impaired erythropoietin generation.

Miller and colleagues[130] investigated the effects of malaria on erythropoiesis by determining the changes in the numbers of early erythroid progenitor cells –

BFU-e and CFU-e in the bone marrow and in the spleen of mice infected with *Plasmodium berghei* at various times post-infection. They found a decline in the levels of BFU-e after the second day of infection, reaching significantly low levels by the seventh day; while the CFU-e was already low by the second day post infection followed by a transient rise and then a fall by the ninth day (Figure 7). They proposed that despite the presence of sufficient levels of erythropoietin, and increased demands for erythrocytes, *plasmodium* infected mice cannot sustain erythropoiesis[130]. In a Gambian case control study which compared anaemic children aged 18 – 36 months with and without malaria supplemented with stable isotope-labelled iron[129], high levels of erythropoietin were found on day one and reduced by day 15 which was inversely related to the levels of STfR, suggesting that iron re-mobilisation in the reticuloendothelial system rather than erythropoietin might be the limiting factor for erythropoiesis[129].

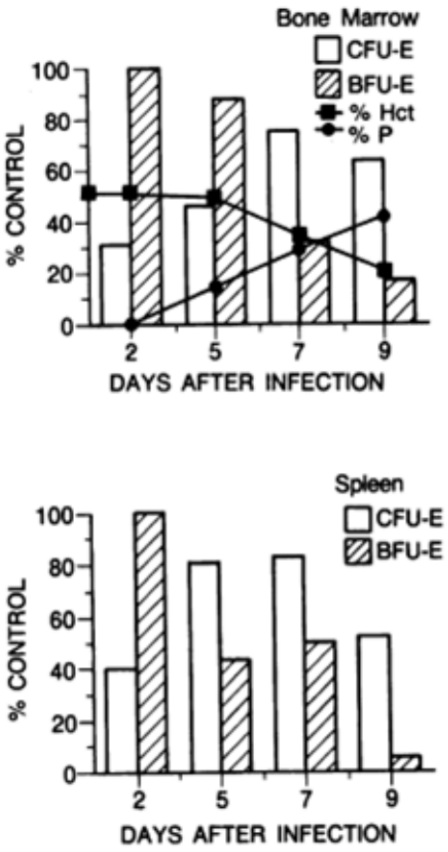


Figure 7: Relative numbers of early erythroid progenitor cells (BFU-E and CFU-E) in the bone marrow and spleen of mice over the course of infection with *P. berghei* (Adapted from Miller KL, Silverman PH, Kullgren B, Mahlmann LJ. Tumor necrosis factor alpha and the anaemia associated with murine malaria. Infect Immun 1989;57(5):1542-6)

3.5 Altered cytokine balance

Malaria associated dyserythropoietic changes could also result from altered cytokine balance during *plasmodium* infection. Jason et al[131] have shown that malaria exerts a pro-inflammatory immune response in children. As in all inflammatory processes, *plasmodium* infection is associated with release of various cytokines which act at various levels of the erythropoietic pathway to cause anaemia. There is strong evidence that human tumour necrosis factor (TNF-α) [130, 132], interferon-γ (IFN-γ)[133-134], and interleukin (IL)-4 and IL-10[133] play significant roles in the initiation and mediation of malaria-associated dyserythropoietic changes. Clark and Chaudhri[132] compared the response to recombinant human TNF by two groups of mouse – one infected with *plasmodium vinckei* and the other group injected with an extract of Coxiella burneti. They found significant erythrophagocytosis and dyserythropoiesis with elevated levels of TNF-α compared to the group of mice not infected with *plasmodium*[132]. In addition to observing similar dyserythropoietic changes in *P. berghei* infected mice injected with TNF-α, Miller and colleagues[130] were able to partially restore erythropoiesis in these mice by treating them with anti-TNF anti serum.

Interleukin-12 (IL-12) contributes to bone marrow dyserythropoiesis and malaria anaemia by simulating $CD4^+$ T cells to differentiate into Th1 subsets which in turn produce IFN-γ. The IFN-γ acts on macrophages to enhance their microbiocidal action and stimulate further production of IL-12 which, through a positive feedback mechanism, modulate macrophage activity with an associated increase in erythrocyte destruction and bone marrow dyserythropoiesis[135]. Administration of recombinant IL-12 enhances erythropoiesis in malaria[136].

Interleukin-10 (IL-10) decreases the production of interleukin-6 (IL-6), IFN-γ and TNF-α. It is associated with reduced production of oxygen radicals and nitric oxide intermediates leading to a decrease in inflammatory response[131]. There are reports that reduced levels of IL-10 is associated with severe anaemia[137]; while a high TNF-α/IL-10 ratio contributes to the reversible bone marrow suppression seen in malaria patients[138-139]. Nussenblatt and co-workers[140] found higher IL-10/TNF-α to be associated with higher haemoglobin concentrations in Ugandan children with acute malaria. Other inflammatory mediators that have been associated with malaria include IFN-γ, IL-4, and IL-6, and neopterin[131, 133-134, 139].

Studies have shown that elevated levels of Th1 cytokines and decreased levels of Th2 cytokines are associated with malaria anaemia, fuelling the strong speculation that in addition to the absolute concentrations of the cytokines, it is the balance between opposing immune and inflammatory responses that determines the clinical characteristics of malaria, including the level of anaemia[131]. Greenberg and colleagues[110] have suggested that the unusually strong and prolonged Th-1 response in conjunction with an inadequately developed Th-2 response may contribute to persistent anaemia after clearance of parasitaemia.

The pro-inflammatory mediator, macrophage migration inhibitory factor (MIF), inhibits BFU-e and other progenitor colony derived units. Injection of *P. chabaudi*-infected erythrocytes or malarial pigment (haemozoin) into mice induced the release of MIF from macrophages to levels that correlated with disease severity[117]. A more recent report provided clearer evidence that MIF is crucial to the pathogenesis of malaria anaemia[116]. Combining *in vitro* studies of erythroid progenitor cells, and *in vivo* studies of MIF knock out mice, McDevitt et al[116] observed a dose dependent inhibition of BFU-e and CFU-e on addition of MIF, TNF-α and IFN-γ both independently and in combination. They also observed a synergistic inhibitory action between MIF and the other two cytokines studied. They demonstrated that MIF does not have cytotoxic effects on the erythroid progenitors; rather they modulate MAP kinase activation which is important in erythroid differentiation. These findings were confirmed in a subsequent *in vivo* study using MIF knock out mice[116].

It is also likely that haemozoin, the malaria pigment from digested haemoglobin, inhibits erythropoiesis either by inducing TNF-α and IL-10 and inhibiting IL-12 production[141] or by direct inhibition of erythropoiesis[142]. Casals and colleagues[142] showed that both isolated haemozoin residues from digested haemoglobin and dilapidated haemozoin inhibit erythropoiesis *in vitro* in the absence of TNF-α. Furthermore, in children with malaria anaemia, the proportion of circulating monocytes containing haemozoin is associated with anaemia and reticulocyte suppression independent of the level of circulating cytokines[142].

3.6 Iron delocalisation and inflammatory conditions

The first suggestion that infections and chronic diseases were associated with hypoferraemia was made by Locke and co-workers following their series of observations in 100 humans and 32 animals in the 1930s[143]. Their work received strong support from data provided by Cartwright and others[144], which showed that hypoferraemia developed within 24–48 hours in patients with acute infections, and could not be relieved by the oral administration of iron. Intravenous administration of iron to such patients was associated with its rapid clearance from the bloodstream after an initial rise, compared with normal controls. Based on these observations, the authors suggested that infection was associated with 'a derangement in intermediate metabolism of iron which diverts the element to some other location and deprives the haemopoietic tissues of iron.' They argued that since absorbed iron was not excreted, the iron in the blood was being diverted from haemoglobin formation by 'important demands elsewhere'. This phenomenon, which came to be known as 'hypoferraemia of inflammation', has since been confirmed by many studies as a normal physiologic response to infection, inflammation and chronic diseases[145-149]. The associated anaemia is usually mild to moderate in severity[149].

To determine the fate of the iron diverted from the plasma during inflammation, Greenberg et al[150] injected radioactive iron intravenously into normal rats and into rats with acute inflammation. The rats with acute inflammation sequestered more iron in their liver than normal rats; whereas the control rats incorporated more iron into their red cells than rats with inflammation. No significant proportion of iron was found in the inflamed muscle. Essentially identical results were found when a larger dose of radioactive iron was injected into two dogs – one with turpentine-induced inflammation and the other without inflammation. In addition, more iron was found in the spleen of the dog with inflammation than the normal dog; and no significant amount of iron was found in the dog's inflamed muscle, the exudate of a sterile abscess or in the excreta. Earlier experiments by Menkin et al.[151-152] had shown that in rabbits experimentally injected with an inflammation inducing agent or infected with *tubercle bacilli* and then treated with ferric chloride, iron accumulated in areas of inflammation and in the caseous areas of the lungs. Greenberg et al[149] however argued that contrary to these reports by Menkin[151] and Menkin et al [152], the low serum iron associated with inflammation was due to diversion to the liver and the

reticuloendothelial tissues rather than to the inflamed tissues. They suggested that Menkin's observations of a relatively high iron load in the inflamed tissue might have been because a smaller quantity of iron was injected into the rats. Later studies have confirmed that iron does not accumulate in inflamed tissues[153], but rather is diverted to the liver and spleen during inflammation[58, 153-155].

In a study utilizing experimental *Neisseria meningitides* infection in mice, Letendre and Holbein[156] presented evidence that the hypoferraemia associated with infection was due to impaired release of iron from the reticuloendothelial system. This, they postulated was as a result of the preferential incorporation of haem iron into intracellular ferritin with very low levels entering the soluble pool; thus reducing available iron for exchange with the circulating transferrin pool. It is now known that inflammation is associated with increased ferritin synthesis[157-158]. In addition, there is strong evidence that the hypoferraemia is cytokine mediated. Among 232 Greek patients with rheumatoid arthritis, serum levels of TNF-α, IL-1ß and IL-6 were significantly higher in anaemic patients than in non-anaemic patients[159], probably through the inhibition of the erythoid units – BFU-e and CFU-e. It is also likely that these pro-inflammatory cytokines mediate hypoferraemia through the induction of IL-10 synthesis, an anti-inflammatory cytokine, which in turn reduces the binding of IRPs to IREs leading to increased ferritin synthesis[160]. It is also interesting to note that IL-6, IL-1α and IL-1ß all induce hepcidin expression, a strong negative regulator of body iron.

3.7 Ferroportin, hepcidin and iron delocalisation

Ferroportin-1 (FPN1) facilitates iron release by macrophages by exporting iron out of the cell, a process requiring either ceruloplasmin (CP), hephaestin or high concentration of transferrin[161-162]. Several human and animal studies have conclusively shown that FPN1 is the major, and possibly the only known, iron exporter in the body[163-165]. This transmembrane protein is abundantly expressed in several iron-handling tissues, specifically on the basolateral membrane of duodenal enterocytes, and in the cytosol of the reticuloendothelial macrophages of the liver, spleen and bone marrow[163-166]. It is essential for intestinal iron absorption[164, 167-168].

Dietary iron is first reduced in the intestinal lumen from the ferric form to the ferrous form by duodenal cytochrome b (Dcytb). The ferrous iron is then

transported across the intestinal apical membrane by divalent metal transporter 1 (DMT1) (Figure 5). Once inside the enterocyte, the iron is exported out of the cell to the portal circulation by ferroportin, assisted by hephaestin or ceruloplasmin, both of which oxidize the iron from the ferrous to the ferric form before it is bound to transferrin. Duodenal FPN mRNA is upregulated by an iron-deplete diet and hypoxia. Paradoxically, ferroportin mRNA contains IRE in the 5´ UTR which is similar to that found in ferritin mRNA, and binds to both IRP1 and IRP2[168] acting as a negative regulator of translation. Thus in conditions of low iron levels in the enterocytes, IRP binds to IRE to inhibit the translation of ferroportin, resulting in a decrease in iron export[163] from the enterocyte to the plasma.

The relationship between this IRE-dependent regulation of ferroportin and the general observation that low body iron upregulates intestinal ferroportin, leading to increased iron absorption, is still not clear. Nonetheless, it is interesting that some reports have shown that TNF-α induces post-translational relocalisation of ferroportin in the enterocyte, independent of hepcidin; and is associated with reduced intestinal iron absorption[155, 169]. It has also been shown that stimulation with TNF-α is associated with a decreased expression of IRE-containing isoforms of DMT1, with no effects on non-IRE containing isoforms[170]. It has been further suggested that IRP-mediated translational control is absolutely required to secure physiological FPN expression in the duodenum, independent of hepcidin expression[171]. Thus it is likely that the regulation of intestinal ferroportin follows more than one pathway: a hepcidin-dependent and a hepcidin-independent pathway (Figure 8). The interaction between these pathways and the ultimate effect on the total body iron would depend on the body's iron needs and the presence or absence of inflammation.

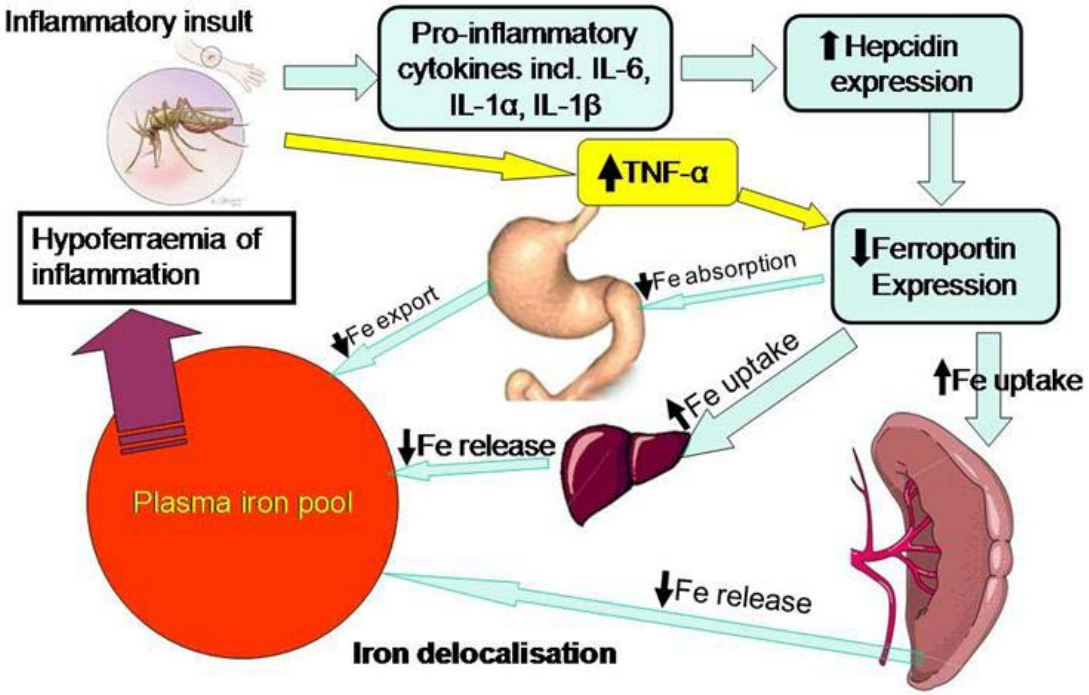


Figure 8: Schematic representation of iron delocalisation

FPN1 also plays a major role in macrophageal iron recycling[165, 172-173]. In the macrophages, FPN1 exports the iron released from the degradation of haem out of the cell, to be oxidized by ceruloplasmin (CP), before coupling to transferrin for onward transport to the target tissues. Mutations in the gene that encodes for FPN1 (SLC40A1; found on chromosome 2q in humans; ala77 to asp substitution) causes FPN1 haploinsufficiency and results in early iron overload in the reticuloendothelial macrophages of affected patients[163].

As already mentioned above, FPN1 is assisted in its iron export functions by other proteins such as CP and hepheastin. Ceruloplasmin (CP) is a multicopper ferroxidase that catalyses the oxidation of ferrous to ferric iron[174], enabling the iron to bind to transferrin. Copper deficient pigs develop an iron deficient anaemia, despite normal or elevated levels of iron[175], which is corrected by intravenous administration of CP[176]. Similarly, aceruloplasminaemia in humans leads to iron accumulation in tissues, including the liver, spleen and pancreas. The ensuing anaemia is thought to result from defective iron release from the RES, and is similar to the hypoferraemia associated with inflammation. Clinically, aceruloplasminaemia has been associated with mild to moderate iron deficiency anaemia[177-179]. Ceruloplasmin is absent in the enterocytes. Hephaestin is a homologue of CP found in the duodenal enterocytes. It has 50% sequence

similarity to CP and, like CP, oxidizes Fe2+ to Fe3+ before export to the portal system.

Post-translationally, FPN1 is down-regulated by hepcidin, a 25-amino acid peptide hormone synthesised in the liver as an 84 amino acid precursor. Hepcidin causes the degradation of FPN1 by binding to it, thus blocking FPN1-mediated transfer of iron across the basolateral membrane of the duodenal enterocytes and into the plasma. Thus hepcidin is a dominant negative regulator of plasma iron. In addition, hepcidin binding to FPN1 also inhibits iron release from macrophages (reviewed in[149, 180].)

Hepcidin is encoded by a 2.5kb gene located on chromosome 19 in humans. Also called liver expressed antimicrobial peptide (LEAP-1), it is a cysteine-rich antimicrobial peptide abundant in the liver and can be isolated from both blood and urine[181-182]. It is categorised as a type II acute phase reactant[183]. Animal studies indicate that over-expression of hepcidin resulted in iron deficiency and severe microcytic hypochromic anaemia; and the administration of synthetic hepcidin induced a decline in serum iron[163]. Hepcidin mRNA was over-expressed in livers of both experimentally and spontaneously iron-overloaded mice; whereas ß2-microglobulin knockout mice fed low iron content diet showed a decrease of hepatic hepcidin mRNA expression[184]. In upstream stimulatory factor 2 (USF2) knockout mice, a complete lack of hepcidin gene expression led to progressive tissue iron overload similar to that found in HFE knockout mice[185], suggesting a regulatory function of hepcidin for both iron absorption and macrophageal iron flux.

Hepcidin is a key mediator of the anaemia of chronic disease (anaemia of inflammation) [186-188]. Hepatic adenomas expressing inappropriately high levels of hepcidin mRNAs were found in six patients with type 1a glycogen storage disease and severe anaemia[187]. The severity of anaemia was related to the size of the tumour, and in one patient resolution of anaemia followed resection of her tumour. It has since been confirmed that hepcidin indeed plays crucial roles in the pathogenesis of anaemia associated with infection, inflammation and chronic diseases[149, 165, 189-191].

IL-6 stimulates the release of hepcidin[191], which in turn inhibits iron absorption and macrophageal iron release[191] leading to hypoferraemia. There are also data showing that hepatic release of hepcidin could be induced by IL-1α and IL-1ß[192]. Lipopolysaccharide (LPS)-induced inflammation increased hepcidin expression in murine liver and in hepatocytes[184]. Treating primary human hepatocytes with LPS led to increase in hepcidin mRNA; whereas treating the cells with anti-IL-6 antibodies blunted this effect[191]. Similarly, in IL-6 knockout mice, hepcidin mRNA did not increase in response to turpentine-induced inflammation; and there was no associated decrease in iron[191].

In summary, iron delocalisation is an important mechanism in the pathogenesis of anaemia of inflammation, and is modulated via a complex interplay of pro- and anti-inflammatory cytokines, and ferro-homeostatic factors principally ferroportin and hepcidin.

3.8 Iron delocalisation and malaria anaemia

Traditionally, the pathophysiology of human malarial disease had been attributed to mechanical occlusion of the vasculature of vital organs of the body (the so called "Mechanical Hypothesis for the pathogenesis of severe malaria") [193]. The mechanical hypothesis relies heavily on the findings at autopsy of the presence of sequestered parasitized erythrocytes in such sites as the brain and placenta. For a detailed review of the mechanical hypothesis, see Newton & Krishna[193]. Many supporters of this school of thought believe that the pathophysiology of malarial disease is unique; and differs from those of other severe systemic illnesses. For example, in a prospective comparison of malaria with other severe diseases in children admitted to a teaching hospital in Ghana, Planche et al[194] reported that the independent predictors of mortality from malaria (Blantyre Coma Score, hyperlactaemia and body mass index) were different from the independent predictors of mortality from other severe illnesses (Blantyre Coma Score, respiratory distress, haematosis and hyperlactaemia). Despite the obvious similarities in the clinical presentations and laboratory findings in both categories of patients (e.g. in both groups, both Blantyre Coma Scale and hyperlactaemia were independent predictors, and the factors associated with hyperlactaemia were similar), these authors interpreted the identified differences to reflect differences in the underlying pathophysiological processes[194].

Advances in the understanding of the pathophysiology of malarial disease have continued to provide strong evidence for the important role of inflammatory mediators in the features and outcomes of human malarial disease. These advances have resulted in a number of hypotheses for the pathophysiology of human malaria. At least two of these hypothesis (the toxin hypothesis and the cytokine hypothesis (see Newton & Krishna) [193]) suggest that inflammatory mediators play important roles in the development and progress of malarial disease. Clark et al[195-196] have reviewed the role of inflammatory cytokines in the pathogenesis of human malarial disease, and argue persuasively that while vascular occlusion could explain some of the findings in malaria, human malarial disease is essentially a consequence of inflammatory cytokines, and bears a lot of similarities to other systemic inflammatory diseases.

The failure of any single hypothesis to explain all the findings in human malarial disease suggests multiple (and possibly related) mechanisms in the pathogenesis of malaria disease. It is however indisputable that systemic inflammation plays a crucial role in the symptomatology and prognosis of human malarial disease. Therefore the pathophysiology of malaria-associated anaemia and that of anaemia of inflammation will likely overlap; and iron delocalisation, which plays a significant role in the pathogenesis of anaemia of inflammation, might also play a significant role in the pathogenesis of malaria-associated anaemia. Indeed, several reports have provided evidence of the role of inflammatory cytokines in the pathogenesis of malaria-associated anaemia[132, 137, 195].

That serum iron is often low in malaria anaemia has been demonstrated in several animal and human studies[115, 197-199], and is the basis for the clinical practice of administering iron to anaemic malaria patients. Decreased peripheral iron levels can co-exist with normal or increased bone marrow iron, and suggests that this hypoferraemia could largely result from macrophageal iron sequestration. In 23 Thai patients with uncomplicated malaria, serum iron was low in 18 subjects, whereas bone marrow iron was normal or increased in 17 subjects during the acute phase and in 15 subjects in the convalescent phase[199]. Similar observations have been reported from The Gambia[115].

A recent report from The Gambia showed that in a cohort of 760 children aged 2-6years followed across a malaria season, the presence of TNF-$_{308}$AA and TNF-

$_{238}$AG was associated with an increased risk of iron deficiency anaemia (IDA); with 41% of children carrying the TNF-$_{308}$AA genotype and 30% of those carrying the TNF-$_{238}$AG had IDA compared to 21% and 22% respectively of children without those genotypes[200]. The TNF-$_{308}$AA genotype was also associated with an increased risk of iron deficiency at the end of the malaria season. As already discussed, TNF-α can induce hypoferraemia by inhibiting FPN independent of hepcidin. Considering that the TNF-$_{308}$AA genotype induces more TNF-α production compared to the other TNF genotypes, this association with iron deficiency at the end of a malaria season supports a role for malaria-induced TNF-α production resulting in hypoferraemia and anaemia[200].

3.9 Pathophysiological, clinical and public health implications

Several mechanisms, including iron delocalisation, contribute to the pathogenesis of malaria-associated anaemia (Figure 6). *Plasmodium* infection initiates inflammatory reactions which produce at least three distinct effects on the haematologic system that ultimately lead to anaemia:

1) Increased destruction of erythrocytes, including immune-specific mechanisms, and also parasite-derived and cytokine-induced oxidative stress that causes oxidative damage to infected and non-infected erythrocytes, resulting in lysis or phagocytosis through non-specific immune mechanisms[201].

2) Reduced erythropoiesis resulting from inflammation-induced bone marrow suppression and a reduced response to erythropoietic signals such as erythropoietin.

3) Reduced iron release from macrophages (iron delocalisation), hampering the iron supply to erythropoietic tissues, and ultimately causing anaemia. Thus, although there may be a strong signal generated for erythropoiesis, such as increased erythropoietin, the response to such signals appears blunted, and even where there is a response, not enough iron is recycled to the erythropoietic compartment for effective erythropoiesis to take place.

One public health implication of the hypothesis of iron delocalisation in the aetiology of malaria anaemia is that it raises doubt about the rationale for administering iron to patients with malaria anaemia. Many of the studies

reviewed above showed that giving oral or intravenous iron during an inflammatory process is not beneficial as the iron is rapidly removed from the blood stream. A recent report from The Gambia compared the absorption of stable isotopes of iron and Hb response in children with post-malaria anaemia or iron deficiency anaemia. The children with post malaria anaemia demonstrated significantly reduced iron absorption compared with those with iron deficiency anaemia (8% vs 28%, p<0.001 at Day 1 post-treatment: and 14% vs 26%, p=0.045 at Day 15 post treatment) [129]. However, the Hb response was significantly greater in the post malaria anaemia group than the iron deficiency anaemia group both at day 15 (p=0.001) and day 30 (p<0.001) [129]. The authors concluded that the likely source of the iron required for this increased Hb response was the remobilisation of previously delocalised endogenous iron.

Thus iron administration during or in the 2 weeks after a clinical malaria episode is unlikely to be beneficial in children with mild or moderate anaemia. The current practice of administering iron to children with mild malaria anaemia would therefore seem questionable[202-203] as the anaemia may result at least in part from delocalised iron rather than absolute iron deficiency. And since the hypoferraemic response that occurs during inflammation is one of nature's ways of restricting the proliferation of invading pathogens[204-207] iron supplementation in the setting of an inflammatory process, such as in malaria, would oppose this natural acute phase response and could tilt the delicate balance between optimal host-pathogen iron requirements in favour of the pathogens[204], predisposing to their increased proliferation, and possibly leading to bacteraemias[205].

There have been several reports of adverse outcomes in children resident in malaria-endemic areas who received iron supplementation in clinical trials. The largest body of evidence for this comes from a Tanzanian clinical trial involving over 24,000 children. In this study, children who received iron and folic acid, with or without zinc, were significantly more likely to die or experience serious adverse events than children who did not receive iron and folic acid[208]. In a subsequent review of iron and malaria interaction, Prentice et al[203] provided evidence from existing literature that routine administration of iron in a malaria-endemic area is associated with adverse outcomes, although the risk is lower in subjects with prior iron deficiency anaemia; hence the need for other

therapeutic options for children with malaria anaemia. In a recent Cochrane review, however, the authors concluded that iron does not increase the risk of clinical malaria or death when regular malaria surveillance and treatment services are provided[209]. Both iron deficiency and malaria cause anaemia in children living in malaria endemic areas[210], and distinguishing which predominates at an individual level is beyond the capabilities of primary and secondary healthcare facilities in these areas. Unfortunately, regular malaria surveillance and treatment is not currently logistically or programmatically feasible in many malaria-endemic areas.

A combined approach to the management of anaemic children in malaria-endemic countries, incorporating malaria prevention strategies and the treatment of both iron delocalisation as well as iron deficiency, is most likely to be effective. At present, children with severe anaemia are likely to benefit from iron supplementation, despite the risks associated with such treatment; however, for children with mild anaemia more emphasis might be placed on iron mobilisation. Since malaria-associated hypoferraemia results from iron redistribution within the storage compartments, rather than an absolute iron lack, early remobilisation of the sequestered iron through the use of anti-inflammatory agents or drugs that disrupt macrophageal iron sequestration could restore serum iron levels, and enhance erythropoietic recovery after malaria.

4 Chapter 4 – Chloroquine as a putative therapeutic agent for malaria anaemia

4.1 Why investigate chloroquine?

The decision to use chloroquine as the anti-inflammatory drug to test for effect in the management of malaria anaemia was an outcome of a long consultation process I had with my supervisors and collaborators. The following drugs were considered: Aspirin (acetylsalicylic acid), ibuprofen, ascorbic acid and chloroquine. Aspirin was quickly dropped because of its ulcerogenic properties and concerns about its safe use in the young children to be recruited in the study. Ibuprofen, ascorbic acid and chloroquine were all thought to be safe to use in children; and the option of a multi-arm study was considered. Such multi-arm study would have enabled us to compare the anti-inflammatory properties of the different agents. However, sample size considerations and the complexity of such a design argued against its adoption. The conflicting evidence about the anti-inflammatory properties of ascorbic acid [211-212] informed its being dropped from further consideration in the study. Ibuprofen is a safe and effective anti-inflammatory agent for use in children. However, it was dropped for chloroquine because of the additional anti-malarial action of chloroquine.

The utilisation of a therapeutic agent with both an effective antimalarial action and the ability to enhance haematologic recovery would provide considerable economic and clinical advantage. The diverse pharmacological properties of chloroquine make it an attractive candidate for investigation. Besides its antipyretic and antimalarial properties, chloroquine acts against a wide range of pathogenic microorganisms, it modulates the immune system, and enhances the phogocytic functions of monocytes and macrophages. Chloroquine also interferes with intracellular iron metabolism and is utilised for its anti-inflammatory actions in such inflammatory conditions as rheumatoid arthritis and other connective tissue disorders.

4.2 Biochemistry of Chloroquine

4.2.1 History and chemical structure

Chloroquine, a 4-aminoquinolene, was discovered by Hans Andersag, a German, in 1934 at Bayer I.G. Farbenindustrie A.G. laboratories in Eberfeld, Germany[213-

[214]. Originally called Resochin, it was renamed chloroquine by U.S. scientists[214]. It is a 7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline – (molecular formula: $C_{18}H_{26}ClN_3$), and has the following structural formula:

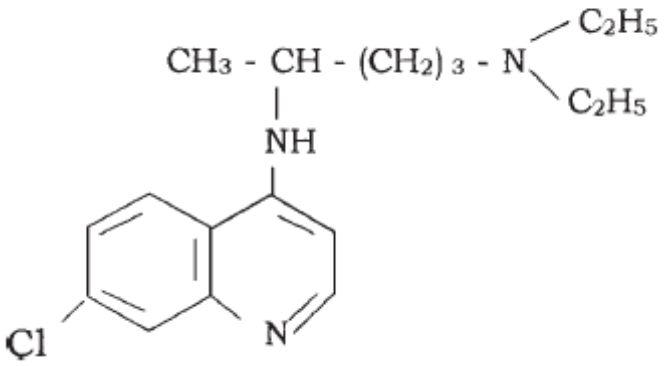


Figure 9: Chloroquine

4.2.2 Chloroquine pharmacokinetics

Chloroquine is a diprotic weak base with pKs of 8.1 and 10.4 at 37°C [213, 215]. It is a lysosomotropic agent (these are chemical substances which are taken up selectively into lysosomes[215]). These substances can enter the lysosomes through one or more of three mechanisms – classical endocytosis, 'piggyback endocytosis' or through permeation. Because of the acidic nature of lysosomes, weak bases such as chloroquine readily accumulate within where they become trapped via protonation[215]. Such 'trapping by protonation'[215] raises the intralysosomal pH.

Chloroquine is rapidly and almost completely absorbed from the gastrointestinal tract, with only a small proportion appearing in the stool[213, 216]. Peak plasma levels of between 55 – 102ng/ml are attained within one to six hours following a single oral dose of 300mg base of chloroquine[217], although there is considerable inter-individual variation, and depending on whether or not it is taken with food[216, 218-219]. The absorption is also very good when given intramuscularly and via nasogastric tube. Chloroquine has a strong affinity for blood constituents particularly thrombocytes and granulocytes which reduces the plasma concentrations[217, 220]. In addition, about 46-74% of chloroquine in the plasma is bound to plasma proteins mainly albumin and α-acid glycoprotein[221-222]. It is also avidly bound to several tissues in the body. In animals, from 200 to 700 times the plasma concentration may be found in the liver, spleen, kidney, and lung[223],

and some melanin-containing tissues such as the retina, the inner ear and hair follicles[224]. The brain and spinal cord, in contrast, contain only 10 to 30 times the amount present in plasma[223].

Excretion of chloroquine is quite slow, but is increased by acidification of the urine. Chloroquine undergoes appreciable degradation in the body by N-de-ethylation[213]. The main metabolite is desethylchloroquine, which accounts for one fourth of the total material appearing in the urine; bisdesethylchloroquine, a carboxylic acid derivative, and other metabolic products as yet uncharacterized are found in smaller amounts. Slightly more than half of the urinary drug products can be accounted for as unchanged chloroquine.

Chloroquine is eliminated slowly from the body and can be detected in the urine for more than a year after intake [225]. It has a multiexponential elimination pattern[217, 226]; the initial elimination phase with a half life of three to six days is followed by a slower phase with a half life of 12-14 days. Terminal elimination half-lives of chloroquine and its metabolite diethylchloroquine of up to two months have been reported[217, 227], although it is thought that this half life is of minor importance in the elimination of the drug from the body. Between 45 and 56% of the total dose of chloroquine is eliminated from the urine within 3-13 weeks and about 8-10% are eliminated in the faeces[225].

4.2.3 Chloroquine pharmacodynamics

Chloroquine is primarily used as an antimalarial drug. It is a schizontocidal drug which is effective against the asexual forms of all the four species of plasmodium that cause malaria in man – *P. falciparum, ovale, vivax* and *malariae*. In addition, it is active against the gametocytes of *P. vivax, ovale* and *malariae* but not against the gametocytes of *P. falciparum*[225]. However, widespread development of resistance to chloroquine by the plasmodium organism in many countries has greatly diminished its usefulness as an antimalarial. Despite decades of chloroquine use, its precise antimalarial mechanism is still unclear. One of the most popular hypotheses is that it acts by inhibiting haem polymerase which detoxifies ferriprotoporphyrin IX in plasmodium food vacuole [228-229]. Chloroquine accumulates in the acid food vacuole of the intra-erythrocytic stage malaria parasite. The *plasmodium* food vacuole degrades ingested haemoglobin to provide the growing parasite with needed amino acids. This process releases haem which in the soluble form is

harmful to biological membranes and inhibits a variety of enzymes. To avert this potential danger, the parasite detoxifies the haem by incorporating it into an insoluble crystalline compound called haemozoin or malaria pigment. It is thought that a component of the plasmodium trophozoites promotes the polymerization of haem to form haemozoin. This haem polymerase activity is inhibited by chloroquine[229]. It has also been suggested that chloroquine forms molecular complexes with plasmodial DNA, causing inhibition of plasmodial RNA synthesis and DNA replication [213, 230-232].

In addition to its antimalarial actions, chloroquine has several other actions on diverse groups of cell types. For example, AtT-20 cells (a mouse pituitary gland tumour cell line) secrete ACTH by cleaving the precursor to ACTH and b-endorphin. These hormones are stored in secretory granules and discharged only in the presence of a secretagogue. Chloroquine blocks the storage of newly synthesized ACTH in the secretory granules and instead diverts it to the outside of the cell[233]. This might relate to its basic properties, which raises the intracellular pH. Chloroquine also competitively inhibits thiol-containing enzymes including succinate dehydrogenase, glutamate dehydrogenase, and alcohol dehydrogenase. This might be the mechanism for chloroquine-induced retinopathy[234]. In adult asthmatics, including those that are steroid dependent, administration of hydroxychloroquine (a derivative of chloroquine) at a dose of 300-400mg/day (max 6.5mg/kg) led to improvement of symptoms[235]. Chloroquine inhibits the replication of a number of viruses such as *Herpes simplex* virus type 1 (HSV-1)[236], human immunodeficiency virus type 1 (HIV-1) and several AIDS related opportunistic microorganisms[237-238]. Chloroquine probably acts at several targets to inhibit HIV such as inhibition of the HIV-1 integrase and Tat-mediated transactivation, and reduces iron stores within cells, which affects reverse transcription[239-241]. Other anti-HIV effects of chloroquine include inhibition of post-transcriptional maturation of gp120[242]. Chloroquine also inhibits the effects of the lethal factor of anthrax[243-244].

Chloroquine reinforces the structural configuration of nucleic acids by binding to it, particularly to the CG sequence of DNA. This prevents mutagenesis and improves DNA repair from the damage induced by alkylating therapy[245-247]. However, some reports have argued that chloroquine is mutagenic rather than anti-mutagenic[248].

4.3 Chloroquine as an anti-inflammatory and immunomodulatory agent

Chloroquine has well recognised anti-inflammatory and immunomodulatory actions. The beneficial effects of chloroquine on chronic inflammatory disorders such as rheumatoid arthritis have been known as far back as the early 1950s[249]. In one of the first recorded randomised controlled trials investigating the anti-inflammatory effects of chloroquine, Freeman[249] showed that after 16 weeks on treatment joint tenderness in patients with rheumatoid arthritis treated with chloroquine showed statistically significant improvement when compared to patients given placebo. The chloroquine group also improved significantly in terms of strength of grip and tenderness and function score[249]. Subsequently, it was confirmed that indeed chloroquine and its derivative, hydroxychloroquine are effective drugs in the treatment of chronic inflammatory diseases.

An unblinded study of 11 asthmatics (four severe symptomatic non-steroid dependent and seven steroid-dependent), who received either hydroxychloroquine per oral at a daily dose of 300-400mg for 28 weeks or placebo showed an increase in symptom scores and lung function[250]. In the steroid dependent asthmatics, there was a reduction in the dose of steroids used from a mean of 383mg/month to 191mg/month at the end of the 28 weeks. A recent review has shown that in patients with systemic lupus erythematosus, chloroquine and hydroxychloroquine provided clinical improvement, prevented lupus flares and increased long term survival of the patients[251]. The anti-inflammatory effects of chloroquine and hydroxychloroquine are also utilised in other dermatological conditions such as porphyria cutanea tarda, cutaneous sarcoidosis, dermatomyositis, Sjögren syndrome, granuloma annulare and erosive lichen planus[252]. In treating these conditions, the maximum recommended doses are 3-4mg/kg/day for chloroquine and 6.5mg/kg/day for hydroxychloroquine.

Panayi et al[253] presented one of the earliest reports suggesting that chloroquine's anti-rheumatoid effects was via an immunosuppressive mechanism. They reported that peripheral blood lymphocytes from patients with rheumatoid arthritis treated with chloroquine exhibited reduced responsiveness to phytohaemagglutinin when compared to peripheral blood lymphocytes of patients with rheumatoid arthritis and of patients with degenerative joint disease treated with soluble aspirin. It has since been confirmed that the anti-rheumatoid effects of chloroquine, at least in part, were mediated through its

inhibition of production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 [254-257], all of which play primary roles in the pathogenesis of rheumatoid arthritis and other inflammatory diseases[254, 258]. Recently it has been reported that chloroquine also inhibits the production of IL-18, an IFN-γ inducing factor which has been implicated in the pathogenesis of systemic lupus erythematosus[259-262].

The multitude of suggested mechanisms through which chloroquine exerts its anti-inflammatory and immunomodulatory functions attests to the profound complexity of the pharmacology of chloroquine; and it is likely that the mysteries of chloroquine will continue to be unravelled well after the present generation.

It has been established that chloroquine inhibits the release of pro-inflammatory cytokines, principally TNF-α, from activated monocytes and macrophages. The mechanism of chloroquine's inhibition of TNF-α production has been intensely studied. While there is no consensus on the precise mechanism through which this inhibition occurs, available evidence indicates that chloroquine acts at different levels in the TNF-α production pathway, depending on the stimulus, to inhibit its release. Chloroquine inhibited release of TNF-α at the pretranslational level in mice peritoneal macrophages[263-264] and PBMCs from normal healthy human volunteers[255, 265-266] stimulated with LPS, without causing direct damage to the cells, by inhibiting TNF-α mRNA expression. The stability of the TNF mRNA was unaffected. Chloroquine also inhibited the release of IL-1ß and IL-6[254-255, 264-265]. Failure of bafilomycin A1 and ammonium chloride, both lysosomotropic agents, to mimic the effect of chloroquine on LPS stimulated TNF-α mRNA levels suggested that endolysosomal alkalization was not responsible for the decreased expression of TNF-α mRNA[255, 267]; although others[265-266] have provided data to the contrary. Karres et al[265] found that chloroquine inhibited TNF-α secretion from the whole blood of healthy human volunteers as well as TNF-α mRNA expression. In addition, these chloroquine effects were mimicked by ammonia and methylamine (both lysosomotropic agents)[265]. Similarly, Weber and Levitz[266] found that chloroquine inhibited TNF-α release from PBMCs from venous blood of healthy volunteers and HIV positive persons stimulated with the fungi *Cryptococcus neoformans* and *Candida albicans* as well as with the mRNA expression. They further reported that these effects were mimicked by other

alkalinizing drugs like ammonium chloride and bafilomycin A1[266]. These two reports provide strong support for the lysosomotropic property of chloroquine being the mechanism through which the inhibition of TNF-α occurs. Rather than being a contradiction, the findings of these two groups might all be correct. As would be seen from other studies reviewed below, it is possible that the mechanism of action of chloroquine depends on the stimuli and on the cell line used. It is also likely that chloroquine acts through multiple mechanisms to bring about its numerous effects.

Jang et al[254], like previous reports, demonstrated chloroquine-induced inhibition of synthesis of TNF-α, IL-1ß and IL-6 in LPS stimulated PBMCs from a Korean blood bank; but in contrast to the earlier studies, they did not find any changes in the level of TNF-α mRNA. These authors suggested that TNF-α synthesis inhibition by chloroquine occurred at a post-translational step, rather than a transcriptional step, by blocking the conversion of cell-associated TNF-α precursor to mature protein, independent of the weak base property of chloroquine. This finding corroborates an earlier report which showed that chloroquine inhibited TNF-α production in LPS stimulated RAW 264.7 macrophages by blocking the conversion of membrane bound 26-kDa precursor of TNF-α to 17-kDa soluble mature protein rather than inhibiting induction of mRNA or production of prohormone, independent of its lysosomotropic property[268]; but enhanced the degradation of IL-1ß and IL-6 mRNA partially through its lysosomotropic property[254].

In a study that examined the effect of chloroquine on the synthesis and metabolism of tumour necrosis factor receptors (TNF-R) in human histiocytic U-937 cells, chloroquine decreased the concentrations of soluble p55 and p75 TNF-α receptors in the culture medium by blocking the conversion of the cell-associated TNF-R to soluble ones and suppressing the release of soluble TNF-R[269]. The disruption of cellular iron homeostasis by chloroquine could also account for its inhibition of TNF-α and IL-6 production[267].

Hugosson et al[270] stimulated PBMCs from venous blood of healthy Swedish adults with TT, PHA and PPD and incubated the cells in different concentrations of chloroquine. While chloroquine did not influence the viability of the PBMCs, it upregulated the number of IL-10 producing cells and downregulated the number

of IFN-γ producing cells, and significantly increased the ratio of IL-10/IFN-γ producing cells[270]. They suggested that chloroquine affects the direction of the lymphocyte stimulation towards anti-inflammatory response by affecting the antigen-presenting cells and the balance between pro-and anti-inflammatory cytokines rather than inhibiting cytokine production[270]. Fas receptor (also called tumour necrosis factor receptor superfamily, member 6 (TNFRSf6)) is a cell surface protein involved in the control of lymphocyte apoptosis[271]. They promote apoptosis through assembly of death-inducing signalling complex (DISC) and subsequent activation of caspase-8 (a member of the cysteine-aspartic acid proteases family of cysteine proteases, which play essential roles in apoptosis, necrosis and inflammation[272-273]). Bcl-2 is an anti-apoptotic member of the Bcl-2 family of mammalian genes that control mitochondrial outer membrane permeabilization (MOMP). Over-expression of the Bcl-2 gene blocks the apoptotic death of pro-B-lymphocyte cell line[274]. Treatment of *Plasmodium yoelii*-infected BALB/*c* mice with chloroquine resulted in a significant increase in apoptosis of B and T lymphocytes via a Fas-mRNA expression independent mechanism associated with downregulation of Bcl-2 expression[275]. Paradoxically, these authors reported upregulation of TNF-α mRNA expression and downregulation of the anti-inflammatory cytokines IL-10 and transforming growth factor-b (TGF-b) expression in the *P. yoelii* infected mice [275].

The Raf-MEK-ERK pathway is part of the mitogen-activated protein kinase (MAPK) cascade, a molecular signalling network that controls the growth, proliferation, differentiation and survival of virtually all cell types[276-277]. The Raf/MEK/ERK pathway begins with the activation of Ras (a small GTPase) and ends with the activation of ERK (a serine/threonine kinase). The activated ERK is able to phosphorylate several substrates in the cytoplasm and the nucleus, and can regulate gene expression[277]. The MAPK cascade is very complex (for example, MAPK activation in macrophages differs from one cell line to another[278]) and much of the physiologic functions remain to be elucidated[276]. There is a growing body of data suggesting linking the MAPK system particularly Raf/MEK/ERK to the modulation of the macrophageal inflammatory processes. Soluble inhibitors of the MERK pathway inhibited the production of TNF-α by human monocytes stimulated with LPS[279-282]. Similar mechanisms have been found to regulate the production of IL-1ß and IL-6 [281-282]. Weber et al[283] studied the effect of chloroquine on the MAPK signalling pathways using LPS stimulated

PBMCs from healthy human volunteers. They found that chloroquine blocked the phosphorylation of ERK by blocking the activation of Raf and MEK kinases; and suggested that inhibition of the Raf-MEK-ERK pathway could be a potential mechanism of chloroquine's anti-inflammatory effects[283].

Chloroquine can exert different effects on different cell types exposed to the same stimulus. For example, chloroquine induced the production of reactive oxygen species (ROS) in a time and dose-dependent manner in human astroglial cells but not in microglial or monocytic cells under similar experimental conditions[284]. Similarly, chloroquine induced expression of pro-inflammatory cytokines, such as lymphotoxin (LT)-ß (also known as tumour necrosis factor-beta), TNF-α, IL-1ß , IL-1α, and IL-6, by human astroglial cells by selectively inducing the production of ROS in astroglial cells but not in cells of monocytic origin[284-285].

Phospholipase A_2 mediates inflammatory processes hydrolyzing the sn-2 acyl bond of phospholipids to release arachidonic acid and lysophospholipids. The arachidonic acid is in turn modified by cyclooxygenases to produce eicosanoids including prostaglandins and leukotrienes which are potent inflammatory mediators[286]. Inhibition of phospholipase A_2 will block the production and subsequent release of the associated mediators. Chloroquine suppressed phospholipase A_2 and also inhibited the release of catecholamines from isolated bovine adrenal medullary cells[287], and from mice peritoneal macrophages[264], giving chloroquine an immunomodulatory function [288-289].

Histamine is a recognised inflammatory mediator. Chloroquine blocks the actions of endogenous as well as exogenous histamine in guinea-pigs[290], blocks histamine induced bronchoconstriction in animal models[291] and decreases antigen induced bronchoconstriction in guinea pig trachea[292]. Infusion of chloroquine in 22 healthy adult volunteers was associated with dose-dependent dilation of the vein on the dorsum of the hand[293]. The degree of venodilation was reduced by co-infusion of histamine 1 and 2 antagonists suggesting a role for histamine release in chloroquine-induced venodilation[293]. Degradation by histamine N-methyl transferase is the principal mechanism for histamine metabolism in many animals including man[294-295]. Unlike diamine oxidase which is localised to certain tissues, histamine N-methyl transferase is uniformly distributed throughout the

body[296]. Human and animal studies have shown that chloroquine is one of the most potent inhibitors of the activity of histamine N-methyl transferase[297-298].

4.4 Chloroquine and iron metabolism

The role of chloroquine in iron metabolism is still poorly understood; but there is compelling evidence that many of the effects of chloroquine on organisms result from its interference with intracellular free iron which deprives the organisms of the iron needed for metabolism. Chloroquine inhibits the growth of *Legionella pneumophilia*[299], *Histoplasma capsulatum*[300] and *Franscisella tularensis*[301] by limiting iron availability in the phagolysosome. Other organisms that are inhibited by chloroquine through iron deprivation are HIV-1 and HSV[241-242, 302]. However chloroquine inhibition of *Cryptococcus neoformans* is independent of iron deprivation[303]. Chloroquine, being a weak base, accumulates in acid intracellular compartments raising the intracellular pH. Legssyer and co-workers[304] have shown that chloroquine significantly reduced incorporation of iron into the liver, spleen and alveolar macrophages of animals loaded in vivo with iron dextran[304]. They assessed the haematological parameters and iron load in male Wister rats that had either been loaded with iron using iron dextran, or made iron deficient by being fed with iron depleted diet over a 5 week period. Chloroquine was administered to these rats beginning one week prior to iron-loading or depleting schedule till the end of the experiment. The uptake of iron by the bronchoalveolar macrophages after iron dextran loading in rats treated with chloroquine was about 400% lower than in rats not treated with chloroquine.

Chloroquine inhibits the uptake of radioactive iron in a dose dependent manner in both neuronal and glial cells *in vitro*, and significantly reduced nitrite release in primary cultures of macrophages from iron loaded rats treated with chloroquine[304]. Mobilization of iron from transferrin and ferritin depends on an acidic environment. At higher pH, iron remains bound to transferrin, and therefore unavailable. Chloroquine therefore interferes with intracellular free iron availability without affecting the level of iron complexed into organic molecules[305]. Intracellular ferritin iron is used for haem synthesis through a process requiring proteolytic ferritin degradation in a lysosomal-like compartment[306]. Chloroquine by raising the pH of the monocytes, limits iron availability.

4.5 Chloroquine and post malaria anaemia

Is there a role for chloroquine in improving the erythropoietic response post malaria? As has been shown above, chloroquine has a plethora of actions on a host of body systems. It is obvious that a lot still remains to be discovered about chloroquine effects. However, in determining the potential role of chloroquine in enhancing haematologic recovery post-malaria, it will be important to consider its antimalarial actions, its effect on iron metabolism, and its anti-inflammatory and immunomodulatory properties. While there is consensus that the aetiology of malaria anaemia is multifactorial, the principal mechanism will remain speculative. Consequently it will be difficult to determine which of the actions of chloroquine will predominate during a malaria episode, especially given the sometimes apparent paradoxes in its activities.

Chloroquine could directly affect haematologic recovery after a malaria episode through its potent anti-inflammatory and immunomodulatory actions, anti-macrophageal iron loading effect, anti-malarial effect (despite widespread resistance), and its effect in restricting release of transferrin and ferritin iron. Figure 10 below highlights the possible sites chloroquine can act to blunt malaria-induced erythropoietic insult and enhance erythropoietic recovery.

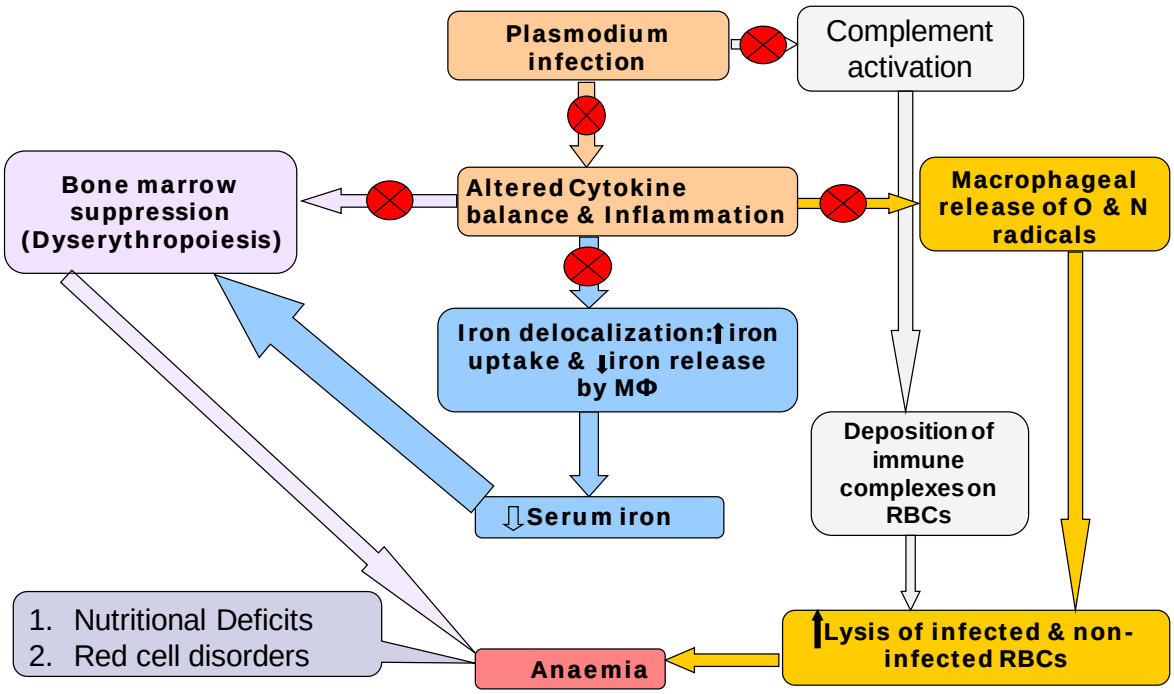


Figure 10: Potential sites of action of chloroquine in enhancing erythropoietic recovery post-malaria

Chloroquine is a weak base and accumulates in acidic intracellular compartments raising the intracellular pH. Such intracellular alkalinisation impedes iron sequestration by the reticuloendothelial macrophages resulting in a reduction in the degree of iron delocalisation and hypoferraemia of inflammation. The 'rescued' iron can then be made available for erythropoiesis.

Malaria induced inflammation is associated with release of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1ß and IL-6 which mediate malaria-associated dyserythropoiesis, iron delocalisation and production of macrophageal oxygen and nitrogen radicals which causes haemolysis of both parasitised and non-parasitised erythrocytes. By inhibiting the release of these pro-inflammatory cytokines and upregulating IL-10 secretion (an anti-inflammatory cytokine), chloroquine can blunt the inflammatory process and reduce the associated insult to the erythropoietic system. Similarly, chloroquine can impede complement activation and the resultant deposition of immune complexes on the erythrocytes sparing the red cells from complement-mediated haemolysis. This is particularly important for non-parasitized erythrocytes which account for about 90% of red cell loss during malaria[88-89]. Thus the anti-inflammatory and immunomodulatory properties of chloroquine could advantageously reduce malaria-induced haemolysis and allow for a faster haematologic recovery.

Ongoing sub-patent infection has been shown to be a factor in continuing anaemia after successful treatment for malaria[125, 307]. Long term chloroquine administration could help to clear such sub-microscopic parasitaemia as well as combat the associated persistent inflammation. In addition, chloroquine could also have a prophylactic effect against further clinical episodes of malaria. In 1956, McGregor et al [308] reported that in Sukuta, a sub-urban area of The Gambia near Banjul, where infants were randomised to receive either a weekly dose of 6mg/kg body weight or weekly placebo from birth until they attained the age of 2 years, children given chloroquine prophylaxis had a statistically significant higher Hb compared to the placebo group (12.1g% vs. 9.5g%). Bradley-Moore et al [309] compared the chemoprophylactic efficacies of chloroquine and pyrimethamine in young Nigerian children from birth to two years. The chloroquine group received 100mg chloroquine base weekly until one year of age, and 200mg base weekly until two years of age. A control group received vitamin C weekly for the duration of the study. Another group received

pyrimethamine 3.13mg weekly until one year of age, and then 6.25mg weekly from one to two years. Their report indicated that children who received either weekly chloroquine or weekly pyrimethamine had significantly higher Hb compared to the control group. Children given chloroquine had higher PCV than children given pyrimethamine. In Mali, Delmont et al [310] reported a rise in PCV of children given chloroquine prophylaxis compared to children that received placebo. In Liberia, monthly doses of chloroquine significantly improved Hb compared to chlorproguanil, pyrimethamine and placebo [311]. Similarly, results from chloroquine chemoprophylaxis in pregnant women suggest strong anti-anaemic effects of chloroquine. Salihu and colleagues[312] reported a significant anti-anaemia effect of chloroquine given weekly to pregnant women in Cameroon compared to women not given any prophylaxis, even after controlling for possible confounders. Other studies among pregnant women in Cameroon[313], Burkina Faso[313], Uganda[314] and Thailand[315] all showed significant benefit of weekly chloroquine on maternal haemoglobin levels compared to controls. Co-morbidities during malaria contribute to malaria-associated anaemia. The inhibitory effects of chloroquine on many micro-organisms could contribute to the resolution of malaria anaemia by reducing the incidences of concurrent infections. Since many of the actions of chloroquine are still poorly understood, this drug merits further investigation for any possible role in enhancing haematopoietic recovery post-malaria.

5 Chapter 5 – Study Aims and Hypothesis

5.1 Justification of the study

The recent controversy regarding routine iron supplementation of children in malaria endemic areas has highlighted the need for alternative therapeutic strategies for children with malaria-associated anaemia. This 'iron supplementation controversy' arose from several reports of increased morbidity and mortality from malaria and other infections in children from malaria endemic regions routinely supplemented with iron. The latest and perhaps the most compelling of such reports is the Tanzanian Pemba Trial which involved over 24,000 children[208]. A recent review of the iron and malaria interaction concluded that routine administration of iron in a malaria-endemic area is associated with adverse outcomes, although the risk is lower in subjects with prior iron deficiency anaemia, and in more anaemic children[203]. As has already been shown (see chapter three), the basis for giving iron to anaemic children with malaria is questionable[202-203] as the hypoferraemia associated with malaria anaemia is a consequence of iron delocalisation rather than absolute iron lack[54]. I have also discussed the evidence in chapter three indicating that during acute malaria, there is reduced iron absorption[129] and that at least initially, erythropoietic iron supply is likely to come from reticuloendothelial macrophages rather than iron supplements. A number of studies have found little or no benefit to haemopoeitic recovery of giving iron supplements to children with malaria anaemia compared to other alternative regimens[34, 36, 316-317]. Although a recent Cochrane Review concluded that iron does not increase the risk of clinical malaria or death when regular malaria surveillance and treatment services are provided[149], currently this is not logistically or programmatically feasible in most malaria-endemic areas.

The recent finding from The Gambia that, compared with children with iron deficiency anaemia, children with post malaria anaemia absorbed significantly reduced amounts of stable isotopes of iron but had a significantly greater Hb response [129] indicates that iron administration during or in the two weeks after a clinical malaria episode is unlikely to be beneficial in children with mild or moderate anaemia. While the management of moderate to severe malaria anaemia (Hb <80g/L) is not in contention, there is presently no clear consensus on the management of children with mild malaria anaemia, a prelude to severer

forms of malaria anaemia. Mild anaemia in malaria endemic areas could result from either or both malaria and iron deficiency but distinguishing malaria-induced iron delocalization from iron deficiency is difficult, if not impracticable, in most clinical circumstances. Iron deficiency and iron delocalisation can be distinguished from each other by examining red cell indices particularly MCV and MCH, by measuring erythrocyte ZnPP, or by doing a bone marrow aspirate. Iron delocalisation does not affect MCV, MCH and ZnPP, which are all altered in iron deficiency. In iron deficiency, a bone marrow aspirate will show iron depletion while in iron delocalisation, the bone marrow will show sufficient iron in the presence of low peripheral iron. Since iron supplementation of this group is potentially dangerous[203], there is an urgent need for alternative management strategies for this very common clinical scenario in sub-Saharan Africa. Such interventions would take into consideration the complex pathogenesis of malaria anaemia including the mechanisms of iron flux and macrophageal iron delocalization during *Plasmodium falciparum* infection. It is possible that reduction of malaria-induced macrophageal iron sequestration and inflammation will enhance erythropoietic recovery post-malaria.

5.2 Hypothesis:

I hypothesised that chloroquine will enhance erythropoietic recovery post malaria by interrupting the malaria-induced inflammatory process, and thereby minimize the malaria-induced inflammatory insult to the erythropoietic system and reduce malaria-induced hypoferraemia.

5.3 Aim

The goal of the study is to explore the effect of acute and continuing administration of chloroquine on haemopoietic response after a malaria episode. I hypothesized that the acute loading of chloroquine at treatment (30mg/kg over 3 days) would have an effect on macrophageal iron flux compared to an artemether based regimen; and that continuing chloroquine administration (5mg/kg weekly for 90 days) would have an ongoing anti-inflammatory and antimalarial effect compared to placebo.

5.4 Objectives

1. To examine the effect of long term administration of chloroquine on haemopoietic recovery in children with malaria anaemia after parasitological cure

2. To examine the long term effect on haemopoietic recovery of treating children with malaria anaemia with either chloroquine-sulphadoxine-pyrimethamine or co-artemether

3. To investigate the mechanisms underlying the effects of chloroquine on haemopoietic recovery

The first objective will be tested by comparing the haemoglobin change in the weekly chloroquine arm to the haemoglobin change in the weekly placebo arm. The second objective will be tested by comparing the haemoglobin change in the placebo arm of children initially treated with chloroquine-sulphadoxine-pyrimethamine to the Hb change in the placebo arm of children initially treated with co-artemether. The third objective will be tested by looking at changes in markers of inflammation (neopterin and cytokines), markers of iron status (MCV, MCH and ZnPP) and reticulocyte percent in the weekly chloroquine arm compared to the weekly placebo arm.

6 Chapter 6 – Methodology

6.1 Study Site

The study was conducted in the villages of West Kiang district of the Lower River Region of The Gambia (Figure 11). Fourteen communities took part in 2007 and nine communities in 2008. The West Kiang district (Figure 12) is a rough rectangle at the south bank of the River Gambia, about 80 kilometres from Banjul the capital city. It consists of 35 villages and settlements with a total population of 14,072; about 18% of whom are children aged one to six years (Keneba MRC DSS Database). The UK Medical Research Council (MRC) Keneba maintains a Demographic Surveillance Survey (DSS) database of all the villages, hamlets and settlements in the entire district. The district is poorly accessible due to a poor road network. An earthen road runs through the district from the main trans-Gambian highway in the South to the bank of River Gambia in the North.

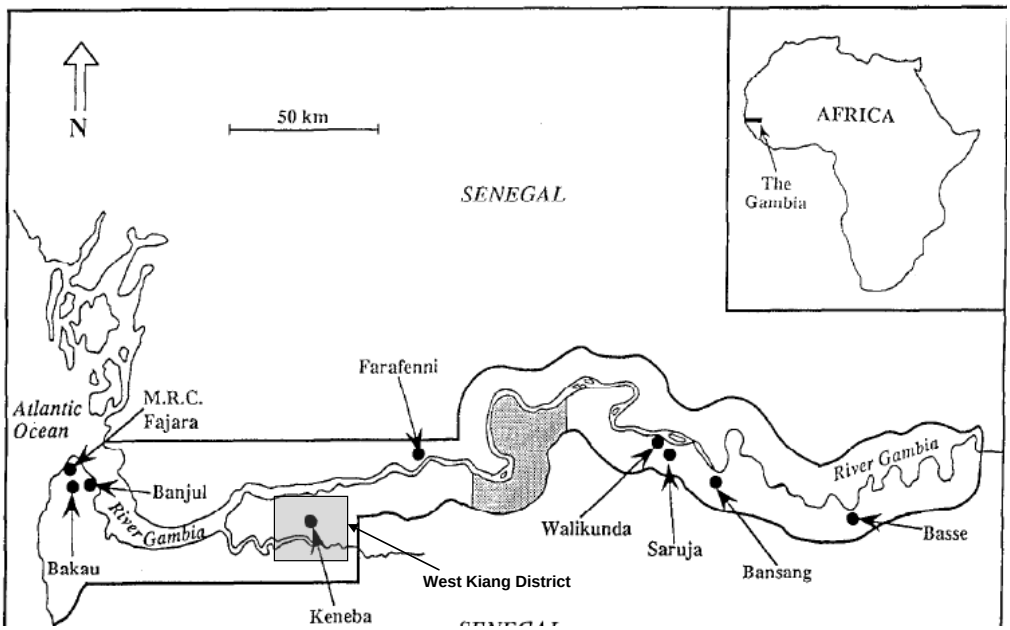


Figure 11: Map of The Gambia showing West Kiang district, the study site. (Adapted from Greenwood BM, Pickering H. Trans R Soc Trop Med Hyg. 1993 Jun;87 Suppl 2:3-11.)

The climate of the West Kiang District is typical of sub-Sahelian Africa with a long dry season lasting from November to June and sometimes July, followed by a relatively short rainy season from July to October[318]. The average annual rainfall in the Gambia varies from one part of the country to the other and ranges from 800mm to 1200mm[319], although there has been a steady decline of rainfall over the past 30 years[318]. Economic activities are limited to subsistence

farming, animal husbandry and occasional small trading. The short rainy season from July to October makes this period the peak season for agricultural activities. The commonly cultivated food crops are rice, millet, sorghum, and groundnuts. The majority of the inhabitants are Mandikas, with fewer numbers of Fulas, Jollas and Wollofs. Nearly all the inhabitants are Muslims.

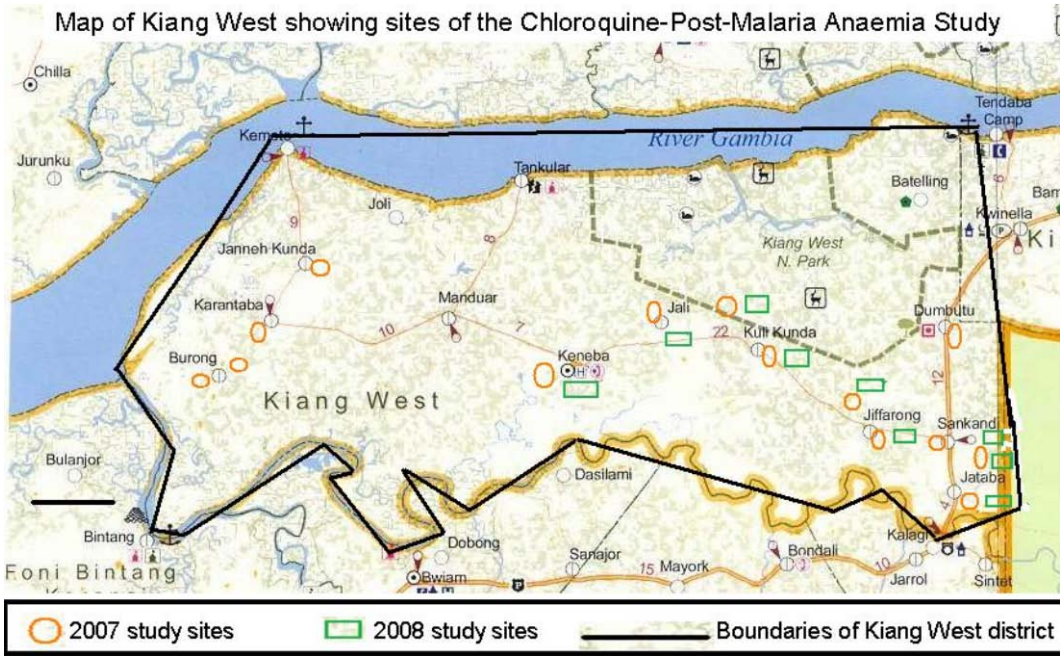


Figure 12: Study Site - West Kiang District, Lower River Division, The Gambia & village groups

6.2 Dietary intake in West Kiang district

The local staple diet consists of rice with some millet, maize, and groundnuts. When in season, vegetables are also consumed in moderate quantities. Animal protein plays little role in the local diet except during festive seasons[22].

Agricultural activities usually peak during the rainy season mainly planting and tending the food crops; and because of the relative scarcity of food stuffs during this period, it is commonly referred to as the 'hungry season'. Malnutrition and iron deficiency are highly prevalent during this period.

At the beginning of this project, we administered food frequency questionnaires to all the eligible participants to obtain an idea of their dietary practices. Figure 13 indicates that the participants' diets consist mostly of locally produced cereals, which are poor sources of iron. In the Gambia, food is usually consumed communally with all the family members eating from a large bowl. Therefore,

although a high percentage of the children reported eating fish at least 4 times a week, the actual quantity consumed may be small (Personal observations). Some of the food items mentioned here like the Baobab juice and porridge are seasonal. Although fruits were not mentioned in this study, when in season, fruits constitute a major component of the diets of children in the study district.

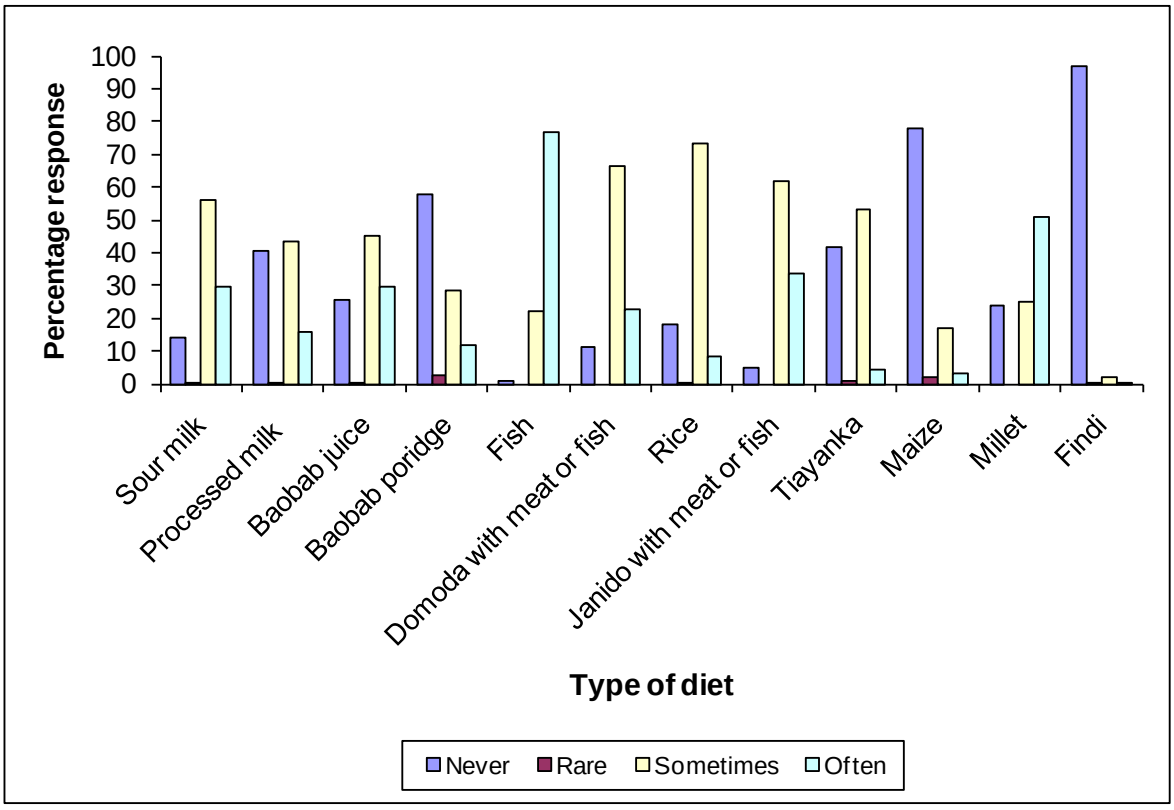


Figure 13: Types of food commonly consumed by the children participating in the study and the relative frequencies of their consumption. ('Never' means that the child never eats the food in question; 'Rare' means that the food is rarely consumed; 'Sometimes' means that the food is consumed at least once a week; Often means that the food is consumed daily or at least more than four times a week

6.3 Clinical malaria and standard of care in West Kiang district

Malaria transmission in the district is seasonal, hyperendemic and follows the rainy season, occurring between July and December (Nweneka CV, Keneba MRC Clinic morbidity audit, unpublished data, Figure 14). The intensity and pattern of malaria transmission during the 'malaria season' (as it is often described), is representative of The Gambia transmission and peaks around October. *P. falciparum* is the dominant species in The Gambia, being responsible for all severe diseases and over 95% of clinical attacks[319]. In the West Kiang district, *P. falciparum* accounts for all cases of clinical malaria. The entomological inoculation rate (EIR) for the district is not known, but inoculation rates of more

than 30 infected bites per person per year has been reported in other parts of The Gambia[320]. Government's malaria-control activities in the district consist of distribution of insecticide treated nets, and case finding and treatment. The malaria incidence among children aged 0-3 years seen at the Keneba MRC clinic 1998 and 2002 was between 3.9% and 7.5% per year. Among a cohort of 1002 children aged 1-6years under active surveillance for malaria in 2003, there were 345 confirmed cases of malaria giving an overall incidence of 34% or an incidence rate of 0.3 episodes of malaria per child per year.

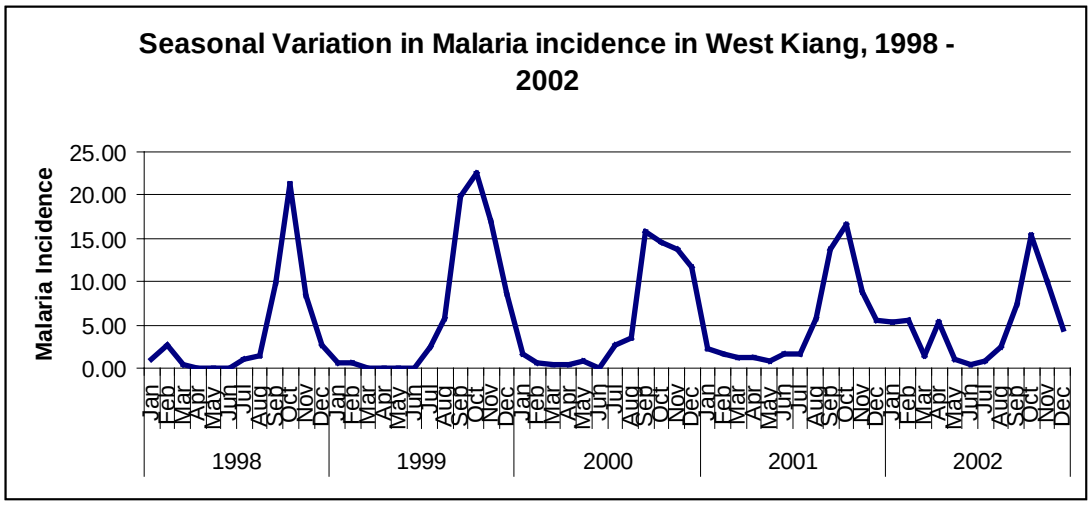


Figure 14: Graph showing seasonal variation in malaria incidence in West Kiang (Nweneka CV, Keneba MRC Clinic Morbidity Audit, 1998-2002, unpublished)

There are two primary health care centres in the district, one of which is the Keneba MRC clinic. The other health centre is the Karantaba health centre, about 20km from Keneba. The Karantaba health care centre is a government-funded health care centre staffed by nurses and nurse assistants. There is also a midwife employed for the centre by the MRC Keneba. There are no laboratory facilities in the centre; therefore clinical conditions are managed empirically based on presenting symptoms and signs. In addition, there are two health outposts – one in Jiffarong village and another in Manduar, both of which are staffed by community health nurses. Each community health nurse oversees a primary health care (PHC) unit. Each PHC unit consists of a group of communities. Each community has a village health worker (VHW) and at least one traditional birth attendant (TBA). The VHWs and TBAs are usually identified by their respective communities and trained by the government on basic first aid and management of uncomplicated labour. They are supervised by the community health worker in charge of their PHC unit.

The Keneba MRC clinic is staffed by three to four doctors at any particular point in time including at least one paediatrician, and several nurses and two midwives. The services offered at the centre include five outpatient consultation clinics, a child-welfare clinic, antenatal clinic and a 24-hour emergency service. The Clinic receives laboratory support from the research laboratory of the Keneba MRC field station. All the services including drug supply are free of charge. Consequently, majority of the inhabitants of the district patronise the Keneba MRC clinic.

In 2007 when the study started, the first line of treatment for malaria in The Gambia consisted of a combination of chloroquine and sulphadoxine-pyrimethamine (CQ-SP). While malaria treatment in the Keneba MRC clinic usually follows confirmation of malaria diagnosis by microscopy, elsewhere in the district, due to a lack of laboratory facilities, malaria is often treated empirically. Following increasing prevalence of resistance to chloroquine in The Gambia, the government in 2008 phased out chloroquine as a first line antimalarial drug replacing it with co-artemether (ACT).

As part of the study protocol, and to enable us to identify all eligible children with malaria, the children needed to be seen as soon as possible after developing a fever. It was therefore necessary to set in place a mechanism to monitor and investigate these children in their homes and to offer appropriate management at village level (active surveillance). This necessitated a disruption of the 'normal' sequence of care-seeking in the study communities as well as improving the standard of care received. For example, the parents were encouraged to seek care from the study team as soon as their children became unwell. The study team worked closely with the VHWs and the nearby health facilities to ensure that all children under surveillance would have access to care and that the necessity to access non-study provided care was minimised.

To effect the surveillance protocol, the study villages were divided into groups of three or four; with each group supervised by a nurse who was resident in one of the villages and provided a 24-hour clinical care to all the enrolled children in that group. With approval from the Regional Health Team (RHT), the various VHWs were required to refer all children aged 12 to 72 months to the resident

nurses. Further description of the activities of the resident nurses is given in the section on 'study logistics'.

6.4 Study Design

The study was initially designed and carried out as a 2x2 randomised placebo controlled trial (Figure 15) in 2007; but with the removal of chloroquine as a first line antimalarial treatment in The Gambia, the study was continued but the design was changed to a two arm double blind randomised placebo controlled trial (Figure 16) in 2008.

In the 2x2 phase of the study, children with malaria were initially randomised to receive a therapeutic dose of either CQ-SP (labelled 'S' in figure 15), or co-artemether (artemether/lumefantrine combination, ACT; labelled 'A' in figure 15). This was an open randomisation as it would have been impracticable to blind the participants and the nurses to the treatment group. Participants who met the eligibility criteria for the second phase of the study were further randomised to receive either weekly chloroquine (labelled '1' in figure 15) or weekly placebo (labelled '0' in figure 15). This second randomisation was double blinded. The participants were then followed up until day 90 or until dropped for other reasons or left the study.

6.5 Study population

The study population consisted of children aged from 12 to 72 months who lived in the selected communities. About 18% of the total population in these communities are aged one to six years.

6.6 Sample size and power calculations

The study was powered to address each of the following two hypotheses:

(i) Post-malarial CQ improves recovery after standard malarial treatment;

(ii) Post-malarial CQ improves recovery after co-artemether treatment, by a mechanism other than its anti-malarial effect in controlling residual parasitaemia.

Hypothesis (i) was tested by comparing the weekly chloroquine versus the weekly placebo group; hypothesis (ii) was tested by comparing the weekly chloroquine versus the weekly placebo arms in children who were initially treated with ACT (A1 vs A0); and A0 versus S0 was used to compare the effect of treating the child initially with CQ or ACT on Hb recovery. Therefore, the same power calculations applied to both hypotheses. As a secondary outcome measure, we also assessed the long term effect of clearing all the parasitaemia with ACT by comparing A0 with S0.

For the two-arm phase of the study (2008), after CQ was withdrawn as a treatment for malaria in The Gambia, the division into 'A' and 'S' subgroups was also dropped. All children with malaria were therefore treated with ACT, followed by either weekly chloroquine or weekly placebo supplementations. Since the power calculation was designed to apply to each arm of the study independently, this change did not affect the testing of the above two hypotheses. The only change was the halving of the calculated sample size for 2007.

Assuming that the standard deviation (sd) for changes in Hb was approximately 15 g/L based on a previous study conducted at our centre[321], the sample sizes for an 80% power and 5% significance level, for various effect sizes would be as shown in Table 1. For us to detect Hb difference of 7.5g/L, we would require 65 subjects per arm or 130 subjects for two arms and 260 subjects for four arms.

Table 2: Sample size calculation

Hb Diff (g/L)	SD diff	No per arm	To**tal all arms**
5.0	3.3	145	580
7.5	5.0	65	260
10.0	6.7	36	144

6.7 Inclusion & Exclusion criteria

6.7.1 Inclusion criteria

Any child in the selected communities was eligible for malaria surveillance if

a) That child was within 12 – 72 months age group;

b) His or her weight-for-height z score was above -3SD during the pre-season anthropometry

c) His/her parents/guardians gave informed consent for the child's participation in the study

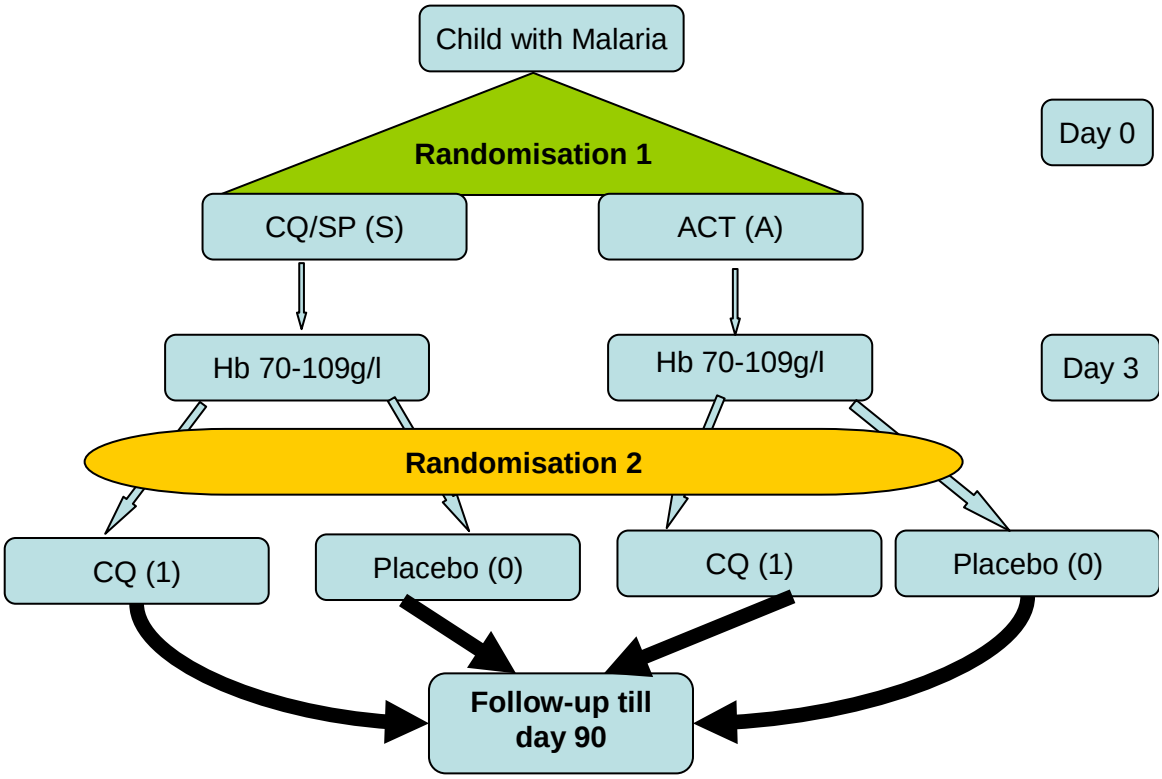


Figure 15: Study design and randomisation protocol in year 1 (2007)

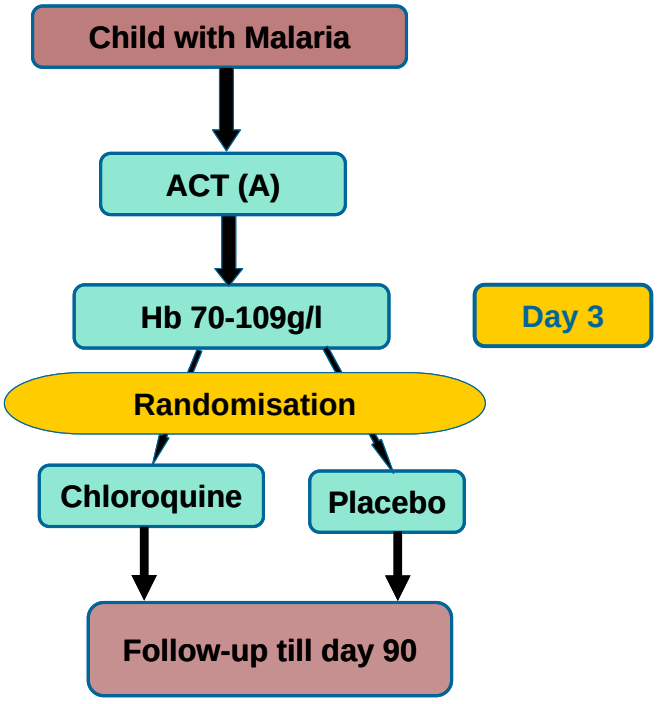


Figure 16: Study design and randomisation protocol in year 2 (2008)

A child under malaria surveillance was eligible for the double-blind placebo controlled randomization if he or she fulfilled **all** of the following criteria:

a) History of fever in the 48 hours prior to presentation or a measured temperature $\geq 37.5^{o}C$ at presentation;

b) Asexual forms of *P. falciparum* in the peripheral blood film of 500/µl or above on day 0 of the study;

c) Hb <110g/l and >69g/l on day three of the study

d) No parasitaemia is detectable on a peripheral blood film by microscopy on the day three blood sample.

6.7.2 Exclusion criteria

Children were excluded from either the malaria surveillance or weekly CQ vs weekly placebo randomisation for any of the following reasons:

a) Inability of the subjects to take oral medications

b) Children who received antimalarial drugs from other sources outside the project team, or other drugs with potential antimalarial or anti-anaemic effects including co-trimoxazole, haematinics, and multivitamins.

c) Failure to clear parasites by day three post treatment

d) Presence of features of severe malaria as defined by WHO[17], with the exception of anaemia and parasite density

e) Children who have urgent need for blood transfusion as indicated by the presence of tachypnoea, tachycardia & gallop rhythm, and tender hepatomegaly

f) Children with known haemoglobinopathy

g) Enrolment in another research project

In addition, children who were already randomised to weekly CQ or weekly placebo and are being followed up were withdrawn from the study if

a) They developed a second episode of malaria during follow-up

b) They developed life threatening symptoms or more severe disease during follow-up

c) They developed serious adverse reaction to study medications.

d) They received any antimalarials from other sources outside the project team, or other drugs with potential antimalarial or anti-anaemic effects including co-trimoxazole, haematinics, and multivitamins

e) Their Hb fell below 70g/L

f) They left the village and were not likely to return for at least 14 days (i.e. two supplementation cycles)

All subjects who withdrew from the trial for medical reasons were visited regularly by the resident nurse until all clinical concerns were resolved. In addition, they continued to benefit, like all the other children in the study villages, from the clinical care provided by the resident nurses. Subjects who withdrew for other reasons were not followed up although they were not excluded from seeking medical care from the project team. No replacement was made for withdrawn subjects. Our inability to achieve the proposed sample size in the first year necessitated a second round of the study. This will be discussed further in the results section.

6.8 Clinical Case Definitions

For the purposes of this study, the following case definitions were used:

1. Fever: axillary temperature of 37.5°C or above.

2. Malaria: For the purposes of commencing treatment, malaria was defined as fever plus a positive malarial dipstick; but to be eligible for the double-blind placebo-controlled randomisation, and subsequent statistical

analysis, the child must in addition have a parasitaemia of at least 500/µL on peripheral blood film microscopy.

3. Pneumonia: A case of pneumonia was defined according to the WHO criteria[322] as cough or difficult breathing with raised respiratory rate for age. Severe pneumonia was defined as cough with chest in-drawing (chest in-drawing being defined as the lower chest wall drawing in when the child breaths in). Fast breathing was defined as 40 breathe per minute or more since all the children were aged one year and above.

4. Cough or cold: A child who had just cough of 30 days duration or less[323].

5. Chronic cough: cough that had lasted for more than 30 days[323].

6. Urinary tract infection: This is fever in addition to positive urinary dipstick result[324-326]. A positive urinary dipstick result was defined as a dipstick applied to freshly voided urine, which is positive for leucocytes and nitrites. The dipstick used was Combur^9 Test (Boehringer Mannheim, Germany).

7. Anaemia: This is haemoglobin concentration less than 110g/L[327]. This was further classified as follows:

    a. Mild anaemia: defined as Hb <110g/L and >79g/L

    b. Moderate anaemia: defined as Hb <80g/L and >50g/L

    c. Severe anaemia: Hb ≤50g/L

8. Diarrhoea: passage of three or more watery or loose stools over a 24-hour period[328].

9. Acute bloody diarrhoea (dysentery): presence of diarrhoea with visible blood[328].

10. Malnutrition: Severe malnutrition was defined as weight-for-height Z (WHZ) scores below –3 standard deviations of the National Centre for Health Statistics (NCHS) standard. The WHO growth charts were not used

for the study because these charts were not available when the study was designed in 2006.

11. Severe malaria was defined according to WHO[17] as follows:

    a. presence of *P. falciparum* asexual parasitaemia plus

    b. the presence of one or more of the following clinical and laboratory features:

        i. Prostration (inability to sit upright in a child normally able to do so, or to drink in the case of a child too young to sit)

        ii. Impaired consciousness

        iii. Respiratory distress with deep sighing respiration

        iv. Multiple convulsions

        v. Circulatory collapse

        vi. Abnormal bleeding

        vii. Jaundice

        viii. Haemoglobinuria

        ix. Severe anaemia

12. Iron deficiency: We used three parameters to define iron deficiency:

    a. Mean corpuscular volume (MCV)

    b. Mean corpuscular haemoglobin (MCH), and

    c. Zinc protoporphyrin (ZnPP)

Studies have shown that a combination of these parameters increases the sensitivity and specificity of these tests to identify iron deficiency[329]. The cut off values for the different parameters employed were : 61µmol/mol of Hb for ZnPP[129], 73fl for MCV[330] and 25pg for MCH[330]. The value for ZnPP has a sensitivity of 66-76% and specificity of 97-95% to correctly identify children with low serum ferritin[331] and falls outside the normal ranges for both male and female given by Soldin et al[332].

6.9 Study logistics

6.9.1 Pre-study

Prior to the commencement of the field work and after the ethical approval had been secured, I developed various instruments that would be required for the study (see Appendix 1). These included:

1) Case Record Forms: clinical record forms, laboratory record forms, temperature record charts and anthropometry record forms;

2) Questionnaires: socio-demographic questionnaires (SDQs) and food frequency questionnaires (FFQs);

3) Team training manual and

4) Work flow chart for every team member.

Team Training: The team consisted of five nurses, five field workers and 14 village assistants in 2007, and four nurses, four field workers and 10 village assistants in 2008. Using the team training manual, I organised a three day training workshop for the team members including the village assistants (VA). During this training, the team members were taken through the various aspects of the study including the basis for the study and the expected outcomes. The various activities that were carried out at each stage of the field work were described and explained. The team members had the opportunity to study the various forms and questionnaires. Issues raised on these instruments were considered and adjustments made where necessary. Two days were devoted to piloting the various instruments during which we conducted mock field work using copies of the forms. The SDQs were given to the Field Workers (FWs) to translate to Mandinka; the translated versions were exchanged among the FWs

for back-translation into English such that each FW back-translated another FW's translation. At the end of this exercise, further adjustments were made as needed before the final copies were printed. All the FWs had been trained in conducting anthropometry by the MRC training department; however, as part of the mock field work, the FWs practiced the anthropometric procedures using children attending the Keneba MRC clinic, under my supervision to ensure the adequacy of their skills. In addition to general team training sessions, a separate training session was held for the VAs on temperature taking and recording using a digital thermometer. This was facilitated by the RNs under my supervision. The VAs were then required to practice doing this for a whole day at the end of which I inspected their record books. VAs with unsatisfactory performance such as poor technique with the thermometer, inability to read the thermometer or to record the readings correctly, went through the exercise again until satisfactory performance was achieved. Refresher training sessions were held for the team members at the beginning of the study in 2008. In preparation for the field work, we also visited the regional health team at the regional headquarters, Mansankonko to inform them of the study and secure their cooperation. Other stakeholders visited were the village health workers in each of the selected villages, traditional birth attendants and community health nurses working in the study villages.

6.9.2 Intra-study

The field work was conducted over two malaria seasons (2007 and 2008). In 2007, the field work was conducted in 14 villages in the West Kiang District of the Lower River Region of The Gambia (Figure 12) between July 2007 and April 2008. These were Janneh Kunda, Karantaba, Jula Kunda, Burong, Keneba, Jali, Kantong Kunda, Kuli Kunda, Bajana, Jiffarong, Sankandi, Nioro Jattaba, Jattaba and Dumbuto. Low malaria incidence in 2007 necessitated the study being extended for one year. Some villages used in 2007 were excluded in 2008. The excluded villages were: Janneh Kunda, Karantaba, Jula Kunda, Burong and Dumbuto. Two considerations informed the exclusion of these five villages from the 2008 malaria surveillance: 1) in these villages, there were very few (about one or two) or no malaria cases in 2007, and 2) the villages were relatively far from MRC Keneba where the laboratory is situated. It was therefore considered prudent to maximise the available resources by excluding these villages and

concentrating on those villages which are closer to Keneba and where there are higher chances of getting malaria cases.

The study villages were grouped into four (2007) and three (2008) (Box 1). Each group of villages was supervised by a resident study nurse (RN) who resided in one of the villages within that particular group. The RN provided 24-hour clinical coverage to all the children within that group. Each RN was given a motorbike to facilitate mobility. He was required to visit each village under his supervision twice a day – morning and evening. During these visits, he first consults the VA to know if any child needed evaluation. He also uses this opportunity to cross-check the VA's temperature monitoring charts. Thereafter, he drives round the village. During these 'village rounds', any parent who had concerns about his or her child would draw the RN's attention to review the child (see 'Work Flow Chart', appendix 2). Each RN worked continuously for four weeks followed by a one week off-duty.

Box 1: Village groups for the Chloroquine-Post Malaria anaemia study

2007	2008
Group 1:	
Janneh Kunda	Keneba
Karantaba	Jali
Jula Kunda	Kantong Kunda
Burong	
Group 2	
Keneba	Kuli Kunda
Kantong Kunda	Bajana
Jail	Jiffarong
Group 3	
Kuli Kunda	Jattaba
Bajana	Nioro Jattaba
Jiffarong	Sankandi
Group 4	
Jattaba	
Nioro Jattaba	
Sankandi	
Dumbuto	

Village assistants (VAs) were recruited for each village – one VA for each small village and two for larger villages. For greater efficiency, the VAs were recruited from within their respective villages. In addition to conducting a twice weekly temperature check on the enrolled children, they also served as a liaison

between the field team and the villagers. They were required to be on duty 24 hours every day. In the event that a VA needed to travel out of the village for any reason, the RN, in the first instance, was notified and arrangements were made to have a replacement. Each VA had a mobile telephone with which to reach me or the RN in case of an emergency. All the members of the field team were supplied with mobile phone credits each month for recharging their phones. In the event of any emergency after the RN had left the village, the VA called him up on phone to return to the village to attend to the emergency. In the event that due to network problems the RN could not be reached, the VA called me directly. Depending on the nature of the emergency (from the account of the VA), I either tried to contact the RN or travelled to the village to personally attend to the emergency. All treatment was done in the field. Any child requiring further treatment was taken to Keneba and treated at the Keneba MRC clinic, or if necessary, at referral hospitals in Banjul. There were five nurses and five field workers in 2007 and four nurses and four field workers in 2008. The field workers had a pool of four motorbikes in 2007 and three in 2008. Each RN was provided with a cold box with ice packs which were changed every day.

Subjects' selection: The names of all the children aged 12 – 72 months in the selected villages were extracted from the MRC Keneba DSS database. The study team visited each village in July 2007 and June 2008 to invite participation and secure informed consent from the parents/guardians of all the children identified from the DSS database. This was followed by the assessment of the nutritional status of all the children whose parents or guardians gave informed consent. Based on the weight-for-height Z scores, a decision was taken whether or not to place each child under malaria surveillance. Children with a WHZ score >-3 were potentially eligible for malaria surveillance (see inclusion and exclusion criteria, section 6.7, page 76).

Surveillance for malaria: Malaria was identified by both active and passive surveillance. Active surveillance for malaria consisted of the VA located in each village visiting each enrolled child twice a week to measure and document the temperature. Children with temperatures of 37.5°C or above were immediately referred to the RN who screened the child for malaria using rapid diagnostic screening test. Active surveillance was commenced from the beginning of August

in 2007 and 15th of July in 2008 until the last follow-up (April 30th in 2007 and February 27 in 2008).

Passive surveillance: the parents/guardians of enrolled children were advised to monitor the wellbeing of their wards and report to either the VA or the RN or any other team member should they have concerns that the child might be unwell. Such reports were immediately assessed and treated as necessary.

Clinical Evaluation: Once a child has been identified as being unwell by either the parent or the VA, that child was seen by the RN (or the PI if the RN is not immediately available). The clinical evaluation consisted of a morbidity questionnaire which requested for details of presenting complaints, and examination for clinical signs. The result of this evaluation was recorded in the clinical questionnaire. Based on the outcome of this initial evaluation, the RN (or the PI where appropriate) made a provisional diagnosis and clinical management plan. All children with confirmed fever or history of fever had a rapid malaria diagnostic test (malaria dipstick) completed.

The Rapid Malaria Diagnostic Test: Screening for malaria was done in the field by the RN using rapid diagnostic dipstick (Cortez diagnostics, INC, USA). All dipstick positive children were eligible for the initial open-labelled randomisation for treatment with either CQ-SP or ACT; and treatment was commenced based on the result of the dipstick. However, to be eligible for the weekly CQ vs weekly placebo randomization (double blind, placebo controlled arm), the child had to fulfil the clinical case definition for malaria as well as meet all the other inclusion criteria (see section 6.7, page 76).

Clinical Management of Children: All children testing positive to the malaria dipstick were commenced on antimalarials immediately. In 2007, the children with positive dipstick were initially randomised to receive either CQ-SP or ACT. This was an open randomisation and was assigned sequentially as the children were identified. It would have been logistically difficult to blind this phase of the trial. In the 2008 malaria season, all the children were treated with ACT for reasons already explained. All children received treatment for three days. All drugs were administered under direct observation of the RN who waited for at least ten minutes after administering the drugs to ensure that the drugs have been swallowed and was not vomited. If a child vomited the administered drug,

the dose was repeated. If a child vomited the administered drug three times, that child was sent to Keneba for parenteral therapy and was withdrawn from the study. Children with fever also received paracetamol. Children with other co-morbidities were treated as appropriate, avoiding drugs such as co-trimoxazole and haematinics. The treatment protocol was as follows:

1. Chloroquine: 10mg/kg once daily for three days

2. Sulphadoxine-Pyrimethamine: ½ tablet per 10kg body weight (i.e. 250mg of suphadoxine and 12.5mg of pyrimethamine) given once on Day one

3. Co-artemether: A total of six doses given over three days – a stat dose, then eight hours later followed by twice daily dose for the next two days. The number of tablets depended on the weight of the child as follows: five to 14.99 kg – one tablet per dose; 15 to 24.99kg – two tablets per dose; and 25 to 34.99kg – three tablets per dose.

Collection of blood and other samples: Two millilitres of venous blood was collected from all children testing positive to the malaria dipstick on the day of initial diagnosis (day 0). This blood was used to confirm malaria by microscopy as well as conduct other investigations (see below). The children also provided urine and stool samples at day 0. The urine samples were used to run urinalysis as part of the clinical evaluation while the stool was used for stool microscopy to assess the presence of helminths. At day three, all the children (except those dropped after the day 0 venepuncture for severe illness or for other reasons) provided 5mls of blood for repeat blood film for malaria parasites (and other laboratory investigations, see blood sampling schedule, Table 2) to confirm clearance of parasitaemia and to check Hb. Further blood sample collection depended on whether or not the subject fulfilled all the criteria required to continue to the weekly CQ/weekly placebo randomisation phase. Table 2 describes the blood sampling schedule for the 2007 malaria season. The blood sampling schedule for the 2008 malaria season was essentially the same except that days seven and 70 blood sample collection were dropped. Efforts were made to bleed the child on the exact date due; however where this was not possible, a window period of 48 hours before or after that date was allowed within which a child should be bled. If a child missed this window period, that particular blood sampling was dropped and recorded as missing.

Since the study villages were spread across the entire district (Figure 12), a mechanism was put in place to enhance the quality of the samples collected. First, the study villages were divided into groups of three or four (Box 1) with each group under the charge of a resident nurse. The resident nurse was equipped with a motorbike, a cold box and some ice packs. The ice packs were changed daily. The resident nurse visited each study village under his supervision twice a day – morning and evening. Two FWs left every morning (including weekends and public holidays) on motorbikes for the study villages, one to the East and the other to the West. During these daily visits, the FWs took along fresh ice packs to replenish the stock of the RNs, cold boxes to collect samples, and any other supplies needed in the field. On meeting the RN, the field workers collected the spent ice packs, replacing them with the fresh packs, delivered any needed supplies to the RN and transported any samples already collected by the RN back to the MRC research lab in Keneba. In the event that no samples have been collected by the RN by the time the FW arrived, the FW is allowed to 'hang around' for a while in anticipation that there might be samples to transport to Keneba. In the event that the RN collected a sample after the departure of the FW, the RN either called the FW, using a mobile phone, to return to the field for the samples, or personally brought the sample to Keneba. Every sample brought to the lab in Keneba was logged in a book. I monitored the performance of the RNs by conducting spot checks and monitoring the entries in the forms that were returned to the Lab. In addition, a field supervisor also went out to the field on regular basis to monitor the activities of the FWs and the RNs.

Table 3: Blood sampling schedule for 2007 malaria season

Study day	Blood Sample	Volume	Investigations
0	Venous	2mls	FBC, MP, qPCR, ZnPP
3	Venous	5ml	FBC, retic count, MP, qPCR, ZnPP, stored plasma for cytokines
7[†]	Finger prick	Drops	FBC, retic count, MP, q PCR, ZnPP
15	Finger prick	Drops	FBC, retic count, MP, q PCR, ZnPP
30	Finger prick	Drops	FBC, retic count, MP, q PCR, ZnPP
45	Venous	5ml	FBC, retic count, MP, q PCR, ZnPP, stored plasma for cytokines
70[†]	Finger prick	Drops	FBC, retic count, MP, q PCR, ZnPP
90	Venous	2mls	FBC, retic count, MP, qPCR, ZnPP

† Blood was not collected on these days in 2008

Follow-up: A dipstick positive child was reviewed daily by the RN until day three. During these follow-ups (FU), the RN reviewed the clinical status of the subject, and administered the study drugs. Data on adverse reactions to the drugs previously administered were also collected prior to the administration of a new dose. After day three, further FU depended on whether the child achieved parasitological cure by day three (assessed by the absence of malaria parasite in the peripheral blood by microscopy), plus whether the day three Hb fell between 70 g/L and 109 g/L, provided the day 0 parasitaemia level was at least 500/µL. All children meeting these criteria were randomised to receive either weekly CQ at a dose of 5mg chloroquine base per kilogram body weight or weekly placebo.

The rationale for the weekly chloroquine supplementation was to exploit the anti-inflammatory actions of chloroquine in enhancing haemopoietic recovery post-malaria. The maximum recommended chloroquine dosage for managing inflammatory conditions is 3-4mg/kg/day. This high dose has been safely used for long durations in the management of inflammatory conditions although there have been several reports of adverse effects to the administered chloroquine. However, information on safety of such a high dose of chloroquine in the management of malaria associated anaemia is lacking. Therefore, a decision was taken to adopt the prophylactic dose of chloroquine which has been shown to be safe and well tolerated by children.

The weekly CQ/weekly placebo was commenced on day 10 and administered once every seven days. All efforts were made to ensure that the weekly supplementation was administered on the same day of the week as the initial supplementation. However, where this was not possible, the child was given the supplement at the earliest possible date but not exceeding two weeks from the last supplementation. A child who missed two supplementations was dropped from the study. Children participating in the weekly CQ/weekly placebo randomisation were seen on days 15, 30, 45, 70 and 90 unless dropped from the study (see 'exclusion criteria', section 6.7.2), for clinical review and sample collection. There was no day 70 FU in 2008.

Randomisation protocol: As already discussed (section 6.4), this study was initially designed as a population-based 2x2 RCT but was changed in the second year to a two-arm double-blind RCT following the change in malaria treatment policy in The Gambia necessitating the suspension of the chloroquine treatment arms of the trial. Randomisation was carried out in blocks of eight, with the codes labelled from A to H and placed in sequentially numbered, opaque sealed envelopes. The randomisation code was generated by a scientist working in Keneba but not in any other way involved in the study. All the investigators and field team were blinded to the supplement codes which were maintained with the Trial Monitor (Dr Mawmo Jawla at the WHO office in Banjul). To ensure masking, both the CQ and placebo supplements were labelled with the respective randomisation codes by a Team in Fajara (Banjul) supervised by the Trial Monitor before they were transported to Keneba. Once a child qualified for randomisation, the resident nurse pulled out an envelope sequentially to retrieve the randomisation code. The supplement bottle with the same randomisation letter was then retrieved and the child's name and study number written on the bottle. The supplements were kept with the nurses who administered each dose to the child, ensuring that it was swallowed before leaving.

6.10 Monitoring of Adverse Events

Occurrence of adverse event was monitored in all children receiving the study drugs. Each dose of medication, including the weekly supplements, was given under direct observation by the resident nurse. Prior to the administration of the medication, an adverse event form (Appendix 9.1.7) was completed by the RN to assess the reaction of the subject to the previous dose of medications or supplementation given. The symptoms noted were then examined to determine if they are related to the administered medication. Since no adverse events were noted among the study participants, nothing was reported in the results section. The intervention drugs were all well tolerated by the participants. This is not surprising since the drug dosages used were all within the recommended therapeutic and prophylactic doses.

6.11 Ethical considerations

Research Governance: The study complied with the current guidelines for good clinical practice. A Good Clinical Practice (GCP) protocol was developed for the

study which was reviewed by the Clinical Trials Support Manager at the MRC (UK), The Gambia. Compliance with the study protocol as well as with the GCP protocol was supervised by the Clinical Trial Monitor, Dr Mawmo Jawla, Disease Prevention & Control Officer, WHO, Banjul. All the required study instruments were developed prior to the commencement of the study and these were explained to all the members of the research team during the team training sessions and at intervals in course of the study. Compliance with the research protocols by the team members was monitored by the PI during field visits. All children in the study villages who took ill during the period of the study were treated appropriately regardless of whether they were participating in the study or not. Treatment was given to the children according to The Gambian Government treatment guidelines. Bed nets were not distributed to the children in the study because The Gambian government does this every year for all the communities in West Kiang.

6.11.1 Ethical clearance

The Scientific Coordinating Committee of the Medical Research Council Laboratories (UK), The Gambia, and the MRC/Gambia Government Joint Ethical Committee approved the study (see appendix 7, page 179).

6.11.2 Informed consent procedures

Informed consent was secured at different levels. Initially village meetings were held involving all the villagers including the Al-kalo (village head) and the council of elders, members of the village development committee (VDCs) and women leaders. During these meetings, the study was explained to the villagers and what it would entail. All issues raised were addressed before their consent was formally requested to conduct the study in their communities. All the selected villages consented to participate in the study. Next, meetings were held in each village with the mothers only. During these meetings the study was again explained to them, giving more details of what would be the role of the children's parents in the study. This meeting afforded the women, who would be most directly involved with the study, to seek clarifications on any areas of the study that was not clear without feeling intimidated by male presence. The meeting with the mothers was also used as an opportunity to inform the mothers that all those whose children had been identified by the demographic database as eligible for the study would be visited to further discuss the study. Prior to the pre-study anthropometric measurements, Field Workers (FW) visited the

parents or guardians of each potential subject to collect individual informed consents. Each parent/guardian was visited by one FW. It was emphasised to the parent/guardian of these children that their participation in the study was voluntary and that they were free to withdraw from the study, or part of it, at any time without giving reasons. Any decision taken in this regard did not have any relevance to the medical care the subject and the family received at the MRC clinic. A written information sheet (see appendix 5, page 174) on the procedure of the study was read out in the language the parent/guardian could understand (either Mandinka or Fula) and explained to him or her. The parent/guardian was encouraged to ask questions if they wanted to. Thereafter, they were asked to sign an informed consent. Those not literate enough to sign thumb-printed the informed consent paper.

Figure 17: Village meeting in one of the study sites to secure informed consent

Figure 18: A cross-section of women at one of the village meetings

Figure 19: A village meeting in progress during the informed consent process

Figure 20: Consenting the villages in a village meeting

6.12 Laboratory Procedures

Table 2 shows the blood sample collection schedules for the study. All venepunctures were done in the field by the research nurses. Vacutainers were used to collect venous blood samples (days 0, 3, 45 and 90) while microtainers were used to collect fingerprick blood samples (days 7, 15, 30 and 70). There was no bleeding on days 7 and 70 during the 2008 malaria season. The blood samples were transported to the research laboratory in MRC Keneba Field Station in cold boxes containing ice packs. As much as possible, these samples were processed immediately upon arrival; but not later than 24 hours of collection. Table 3 shows the different laboratory investigations conducted on samples collected. Zinc protoporphyrin was measured with a fluorometer (Aviv Biomedical, Lakewood, NJ, USA). Full blood count and reticulocyte count were measured using a Cell Dyne (Abbott Laboratories. Abbott Park, Illinois, U.S.A.).

Table 4: Laboratory investigations conducted

Investigations	Specimen	Days collected[†]	Comments
Microscopy for malaria parasite	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess parasitaemia
Erythrocyte zinc protoporphyrin	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess iron status
Full blood count	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To monitor HB changes
Reticulocyte count	Blood	Days 3, 7, 15, 30, 45, 70, 90	To monitor BM response
Cytokines (IFN-g, TNF-a, MIF, IL-6, IL-10, IL-12)	Plasma	Days 3 & 45	To monitor inflammatory response
Quantitative PCR	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess sub-microscopic parasitaemia
Urinary neopterin	Urine	Days 3, 15, 30	To assess macrophageal activation
Stool microscopy	Stool	Day 0	To assess helminthic infection

[†] No blood was collected on days 7 and 70 in 2008

6.12.1 Microscopy for malaria parasites

Thick blood films were prepared by dropping approximately 10µl of whole blood from the microtainer or vacutainer onto a microscope slide already pre-labelled with the subject's number and the date of study. This spot blood was mixed

evenly and allowed to air-dry overnight before being stained with Giemsa. Another thick film was similarly prepared at the other end of the same slide. The Giemsa stained blood film was then read by light microscope with x100 magnification in oil immersion lens.

The Giemsa stain was prepared by dissolving 3.8g of Giemsa powder in 250mls of methanol by shaking it in a circular motion. After shaking the mixture for two to three minutes, 250mls of glycerol was added and the resulting solution shaken for another 2-3 minutes. Thereafter, the mixture was shaken for two to three minutes every 30 minutes for 3 hours and allowed to stand for two to three days, with occasional shaking. Giemsa buffer was prepared by dissolving 1.0g of disodium hydrogen phosphate (Na_2 HPO4) and 0.7g potassium dihydrogen phosphate (KH_2 PO4) in a litre of distilled water. The pH was then adjusted to about 7.2 with 2% Na_2HPO4 or 2% K_2HPO4 solution. The Giemsa staining was done by pipetting 3-5 ml of the Giemsa stain freshly diluted 1:10 in Giemsa Buffer onto the slide containing the thick blood film placed on a staining bar. After 10 minutes, the slide was gently rinsed in tap water until the films turned blue or grey. The slide was then dried in the upright position. All the slides were read by two experienced slide readers working at the MRC Keneba research laboratory. The slide readers examined 200 high powered fields (hpf) before a slide was declared negative. If parasites were seen, two representative fields were read if the parasitaemia was greater than 100/hpf; if low levels were present, i.e. less than 1/hpf, then 200 fields were read. The results were reported as the number of parasites per hpf or number of parasites per 200 hpf. The parasite density per µl was calculated by multiplying the number of parasites per hpf by 500[333]. The arithmetic mean of the parasite density of the two microscopists is taken to be the final parasite density.

Quality control: All the slides were stored in slide racks. Each slide was read by two microscopists based at the MRC research lab, Keneba. Discrepancies between the two slide readers were resolved by a third more experienced microscopist from the malaria research unit in MRC Fajara (The Gambia). The absolute parasite count was $\log_{10}$ transformed; and a discrepancy is said to exist if the difference in reading between the two primary slide readers exceeds one log-unit (Table 5). The parasite count of the microscopist whose reading is closest to the reading of the third microscopist is taken as the correct reading.

This reading is then added to that of the third microscopist and the arithmetic mean calculated to get the parasite density for that particular subject. Prior to statistical analysis, the parasite density was log-transformed since this variable was skewed.

Table 5: An example of a quality control table for the microscopy

		Microscopist 1				Microscopist 2					
WKN	Study Day	HPF	Count	per µL	Log1	HPF	Count	per µL	Log2	Log1-Log2	Comments
04-031-009V	0	1	1500	750000	5.875	1	1700	850000	5.929	-0.054	ok
10-018-009B	0	1	1500	750000	5.875	1	908	454000	5.657	0.218	ok
03-048-033B	0	1	400	200000	5.301	1	540	270000	5.431	-0.130	ok
11-023-016E	0	1	510	255000	5.407	1	536	268000	5.428	-0.021	ok
10-008-024A	0	50	664	6640	3.822	1	484	242000	5.383	-1.561	repeat
02-018-013X	0	1	267	133500	5.125	1	317	158500	5.200	-0.074	ok
11-029-080K	0	10	65	3250	3.511	1	276	138000	5.139	-1.628	repeat
29-054-018G	0	1	1250	625000	5.795	1	111	55500	4.744	1.051	repeat
03-067-014C	0	1	100	50000	4.698	1	110	55000	4.740	-0.041	ok
05-027-027F	0	1	200	100000	5.000	1	101	50500	4.703	0.296	ok
06-006-016Q	3	200	11	27.5	1.439	200	0	0	0.000	1.439	repeat
11-014-035T	3	200	7	17.5	1.243	200	0	0	0.000	1.243	repeat
01-039-008-W	0	1	70	35000	4.544	100	100	500	2.698	1.845	repeat
01-042-022-U	0	100	38	190	2.278	100	3	15	1.176	1.102	repeat
02-008-001-V	0	1	60	30000	4.477	200	0	0	0.000	4.477	repeat
02-008-023-R	90	1	26	13000	4.113	200	0	0	0.000	4.113	repeat
02-008-023-R	0	1	300	150000	5.176	200	0	0	0.000	5.176	repeat
02-008-023-R	70	200	0	0	0.000	100	11	55	1.740	-1.740	repeat
02-017-029-B	3	200	0	0	0.000	200	200	500	2.698	-2.698	repeat
02-022-001-H	0	50	250	2500	3.397	200	0	0	0.000	3.397	repeat

Legend: HPF = number of high-power field read; Count = total number of parasites counted; per µL = number of parasites per micro-litre

6.12.2 DNA Extraction and Quantification

Although slide microscopy has been the gold standard for diagnosis of malaria and measurement of parasitaemia for over a hundred years, it presents many problems and has inherent limitations. Sensitivity is compromised by the very limited volume of blood that can effectively be viewed on a slide since only a maximum of 200 fields are screened in most protocols, only about 0.4µL blood is examined by slide microscopy[333-334]. Slide microscopy has predicted limits of detection of about 50 parasites/µL[335] compared to reported values as low as 20 parasites/mL for real-time quantitative PCR[336] (i.e. up to 1000-fold difference). In view of the increasing prevalence of chloroquine resistance in The Gambia, a high prevalence of sub-microscopic parasitaemia was expected among the study participants especially in those treated with CQ-SP. Considering that sub-microscopic parasitaemia could provide a source of ongoing inflammation, it was important to measure this in order to account for any changes in inflammatory parameters observed. Recently, a number of PCR-based methods that detect and quantify malaria parasites have been reported[337-340]. These protocols have been shown to be sensitive in detecting malaria parasites. PCR-based methods were employed in addition to standard microscopy for the quantification of malaria parasitaemia in the cohort studied, allowing the assessment of the presence of sub-microscopic parasitaemia.

6.12.2.1 DNA extraction

DNA was extracted from whole blood stored in EDTA tube at -20°C until extraction. DNA extraction and subsequent quantitative polymerase chain reaction (qPCR) amplification were carried out by MRC Fajara malaria diagnostics team using established protocols[341]. The DNA extraction was done using QIAamp DNA Mini Kit (QiagenTM LTD, UK) according to manufacturer's instructions with some modifications (see Appendix 3). This kit uses a silica-membrane-based nucleic acid purification process and can be used to isolate DNA from blood, plasma, urine and other body fluids. For each sample, 100µl blood volume was extracted and eluted in 50µl of elution buffer resulting in a two-fold concentration of the original sample volume. The quality of the extractions were checked by running 2µl of the extracted DNA from a random set of samples on a 1% agorose gel containing Ethidium bromide and visualizing under UV light. Extracted DNA samples were stored at -20°C.

6.12.2.2 Quantitative Real-Time PCR (qPCR) analysis of samples

Quantification of parasite density was done with an Opticon® 2 Real-Time PCR Machine using the Opticon Monitor™ Version 3.1 software (Bio-Rad Laboratories, Inc., USA). The TaqMan assay which uses a fluorogenic probe to detect a specific PCR product was used. Primers and probes were synthesized by Applied Biosystems, UK. Each assay was run in duplicate wells in a 20µl final reaction volume containing 2.5 µl of extracted DNA, 10µl of Probe PCR master mix, 0.3µM each of forward and reverse primers and 0.075µM of the probe using standard cycling parameters of 95°C for 15 minutes followed by 45 cycles of 94°C for 15s, and 60°C for 1 minute with plate read at 60°C (Table 4). Each run included both PCR and DNA extraction negative controls. A standard curve was produced from 10-fold dilution series (a range of 7 x 10^5 to 0.7 genome copies per reaction) of *P. falciparum* 3D7 parasites. Baseline subtraction option was used to fit the standard curve and the threshold cycle (C_T) for detecting amplification was manually set to an optimized laboratory standard of 0.02 fluorescence units for all assays

Table 6: Details of primers and amplification protocol used for quantitative PCR analysis of samples

Primer	Sequence (5` to 3`) and Reference	PCR Protocol
	Rougemont et al 2004[342]	
Plasmo 1	GTTAAGGGAGTGAAGACGATCAGA	
Plasmo 2	AACCCAAAGACTTTGATTTCTCATAA	95^0C -15mins; (94^0C-15s; 60^0C-60s, Plate Read-60^0C)**X45**
Falcprobe	**FAM**-AGCAATCTAAAAGTCACCTCG AAAGATGACT-**TAMRA**	

6.12.2.3 Generating the qPCR Standard Curve

A standard curve was produced from the dilution series of ring-stage parasites. The equivalent parasite density analyzed per assay at each dilution was 7 x 10^5; 7 x 10^4; 7 x 10^3; 700; 70; 7 and 0.7 parasites respectively for the stock culture, 1:10, 1:100, 1:10^3, 1:10^4, 1:10^5 and 1:10^6 dilutions. The baseline subtraction option was used to fit the standard curve and the cut-off line for detecting the threshold cycle (C_T) was manually set to a laboratory standard which was used for all assays. The C_T value represents the cycle number in which there is the first detectable increase in fluorescence (above a predetermined background threshold) and is used to calculate the starting amount of template DNA in each

sample. The programme automatically plots the C_T values of the dilution series against the parasite count, generating a linear graph, (the standard curve) from which the starting concentration of parasite in the samples is determined. The standard curve gives the slope, the intercept and a correlation coefficient for the assay with the slope being a measure of the reaction efficiency. The detection threshold was fitted in the log-linear phase of the amplification plot with a minimum of five data points on the standard curve (Figure 21) and a correlation coefficient greater than 0.99 which is required to reliably and accurately quantify the starting amount of DNA in the samples (Figure 22).

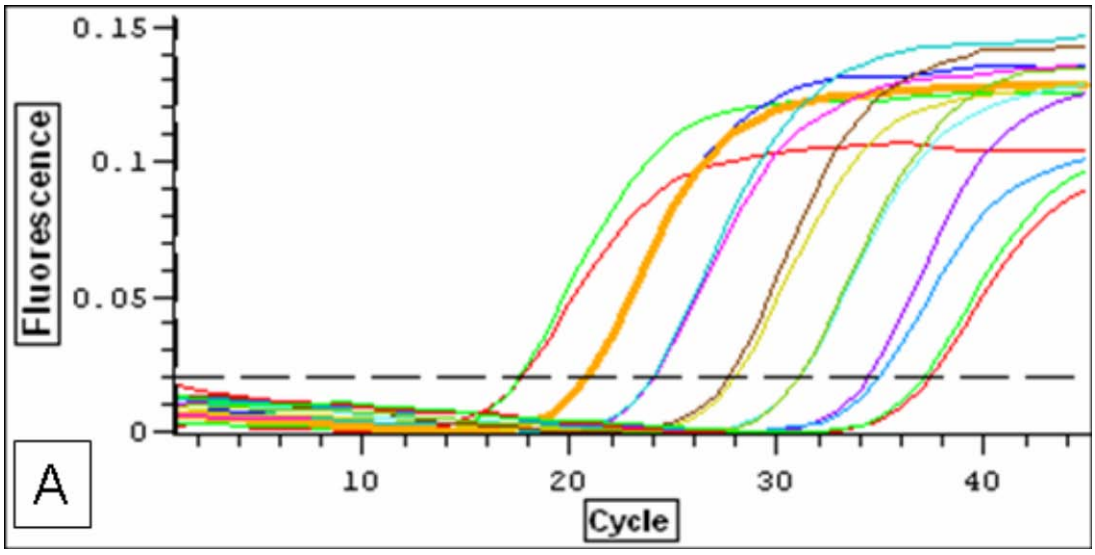


Figure 21: Typical quantitative real-time PCR (qPCR) amplification plots of DNA standards prepared from ten-fold serial dilutions of 3D7 parasite culture (A)

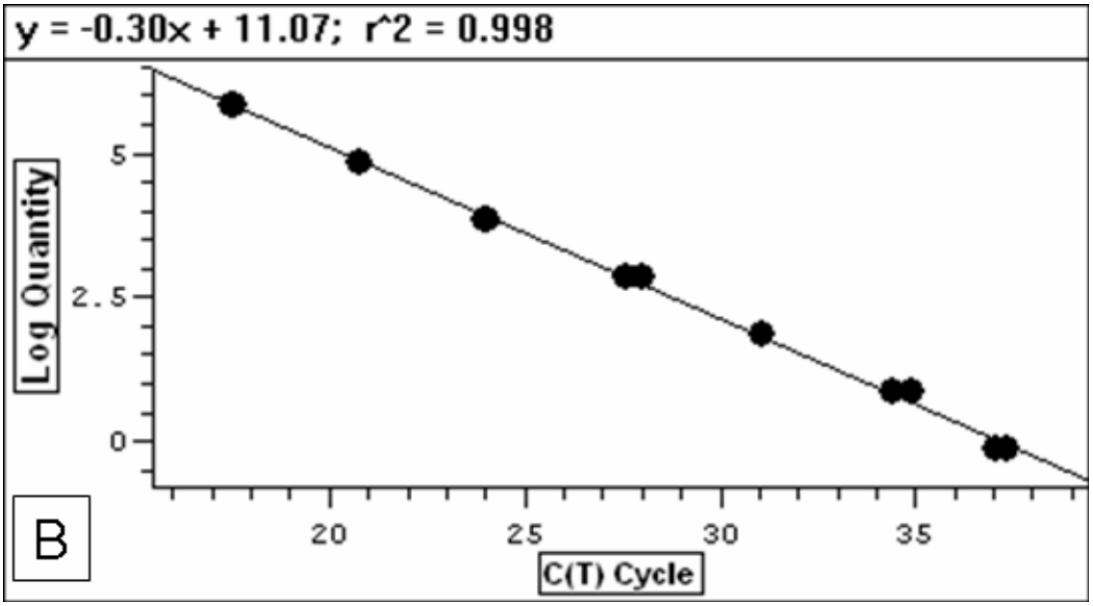


Figure 22: Generation of standard curve (B) for qPCR used to estimate malaria parasite density in blood samples

6.12.3 Cytokine Assays

In 2007, plasma samples were collected for cytokine assay. An EDTA vacutainer tube was used to collect 5mls of venous blood which was transferred to the lab within four hours of collection. On arrival in the lab, 3mls of blood was taken off for FBC, malaria microscopy, ZnPP, retic count, and qPCR for malaria parasite. The rest of the blood was spun and the plasma harvested for cytokine assay. The plasma was stored in -80°C until analysis. In 2008, supernatant from overnight whole blood culture was used to measure plasma cytokines.

6.12.3.1 Blood culture and supernatant preparation

The overnight whole blood culture was carried out using standard methods (Appendix 4). Five millilitres of fresh whole blood was collected in heparinised vacutainer tubes and transported to the laboratory in cool bags containing ice packs within 4 hours of collection. Upon arrival in the lab, 3mls of blood was used to set up the whole blood culture while the rest of the blood was used for other investigations. The whole blood culture was done in a 96-well round bottom plates. The 96-well plate was labelled with the sample number, culture days and the antigens to be added according to a predetermined template (Figure 23). For each donor sample, 150µl blood was dispensed into 18 wells. Eight conditions were used to stimulate the wells – anti-CD3/anti-CD28 (aCD3/aCD28), tetanus toxoid (TT), purified protein derivative (PPD), lipopolysaccharide (LPS), uninfected RBC (uRBC), *Plasmodium falciparum* schizont extract (PfSE), apical membrane antigen 1 (AMA1) pool, and merozoite surface protein (MSP)-1 pool. For each donor, each culture condition was set up in duplicate making a total of 16 stimulated wells. The remaining two wells per donor were unstimulated. RPMI medium was added to these unstimulated wells. After adding the antigens and the RPMI media to the appropriate wells, the plate was sealed and incubated at 37°C in 5% CO_2. Table 7 describes the working and final concentrations of the antigens used. The antigens were procured from the following manufacturers: anti-CD 28 from eBioscience Inc (San Diego, CA, USA); anti-CD3 from BD Bioscience (BD Pharmingen, San Diego, CA, USA) and the LPS antigen from Cayla-InvivoGen Europe (Toulouse, France). The PPD, PfSE, AMA-1 pool and MSP-1 pool were kindly donated by Dr Katie Flanagan (Infant Immunology, MRC (UK) The Gambia), while TT antigen was provided by Dr Pa Tamba Ngum (MRC International Nutrition Group, The Gambia). One of the duplicate wells (labelled as day 1) was cultured overnight while the second well

(labelled day 3) was cultured for three days. The supernatants from the day 1 wells were harvested in the morning following the culture, while those from the day 3 wells were harvested on the third day of culture. All the available supernatants were harvested into pre-labelled tubes which were then stored at -20°C until assayed.

	1	2	4	5	6	7	8	9	10	11	12
Donor 1 day 1→ A	Med	aCD3/28	TT	PPD	LPS	URBC	PfSE	MSP	AMA		
Donor 1 day 3→ B	Med	aCD3/28	TT	PPD	LPS	URBC	PfSE	MSP	AMA		
Donor 2 day 1 → C	Med	aCD3/28	TT	PPD	LPS	URBC	PfSE	MSP	AMA		
Donor 2 day 3 → D	Med	aCD3/28	TT	PPD	LPS	URBC	PfSE	MSP	AMA		
Donor 3 day 1 → E	Med	aCD3/28	TT	PPD	LPS	URBC	PfSE	MSP	AMA		
Donor 3 day 3 → F	Med	aCD3/28	TT	PPD	LPS	URBC	PfSE	MSP	AMA		
Donor 4 day 1 → G											
Donor 4 day 3 → H											

Figure 23: Overnight whole blood culture template

Table 7: Antigens used for stimulation during the overnight whole blood culture

Condition (working concentration)	Storage	Amount added to 150µl of whole blood (µl)	Final Concentration (µg/ml)
aCD3 (100µg/mL)	4°C	7.5	5
aCD28 (100µg/mL)		7.5	5
TT (100µg/mL)	4°C	15	10
PPD (100µg/mL)	4°C	15	10
LPS (10 µg/mL)	-20°C	15	1
RPMI	4°C / RT	15	NA
URBC (200µg/mL)	-70°C	15	20
PfSE (200µg/mL)	-70°C	15	20
MSP Pool (200µg/mL)	-20°C	15	20
AMA Pool (200µg/mL)	-20°C	15	20

6.12.3.2 Cytokine assay

The cytokine assays were conducted using Bio-Plex Pro™ Human Cytokine, Chemokine, and Growth Factors Assays (Bio-Rad Laboratories, Inc, USA) according to the instructions provided by the manufacturers (Figure 24). The assays were performed at the MRC (UK) The Gambia Laboratory in Fajara with assistance from laboratory scientists working with the Infant Immunology Unit.

The Bio-Plex System (adapted from the manufacturer's manual)

The Bio-Plex system has the capability of detecting up to 100 different types of molecules in a single well of a 96-well plate using very little sample through a technology that utilizes multiple fluorescently dyed magnetic beads. A flow cytometer with two lasers and associated optics measure the different molecules bound to the surface of the beads. The resulting fluorescent data is then managed by a high-speed digital signal processor. The basic principle used by this system is the "capture sandwich immunoassay principle" wherein antibodies directed against the target cytokine is covalently coupled to internally dyed beads. After washing the coupled beads to remove unbound proteins, a biotinylated detection antibody specific to an epitope different from that of the capture antibody is added to the reaction to form a sandwich of antibodies around the cytokine target. A streptavidin-phycoerythrin (streptavidin-PE) reporter complex is subsequently added to bind to the biotinylated detection antibodies on the bead surface. Data is acquired from the reaction using a dual-laser, flow-based microplate reader system. After the contents have been acquired by the reader, the internal fluorescence of the individual dyed beads as well as the fluorescent reporter signal on the bead surface are detected by the lasers and associated optics. This identifies each assay and reports the level of target protein in the sample. Intensity of fluorescence detected on the beads indicates the relative quantity of target molecules in the tested samples. A high-speed digital processor efficiently manages the data output, which is further analyzed and presented as fluorescence intensity (FI) and target concentration on Bio-Plex Manager™ software. Figure 24 describes the workflow of the assay procedure.

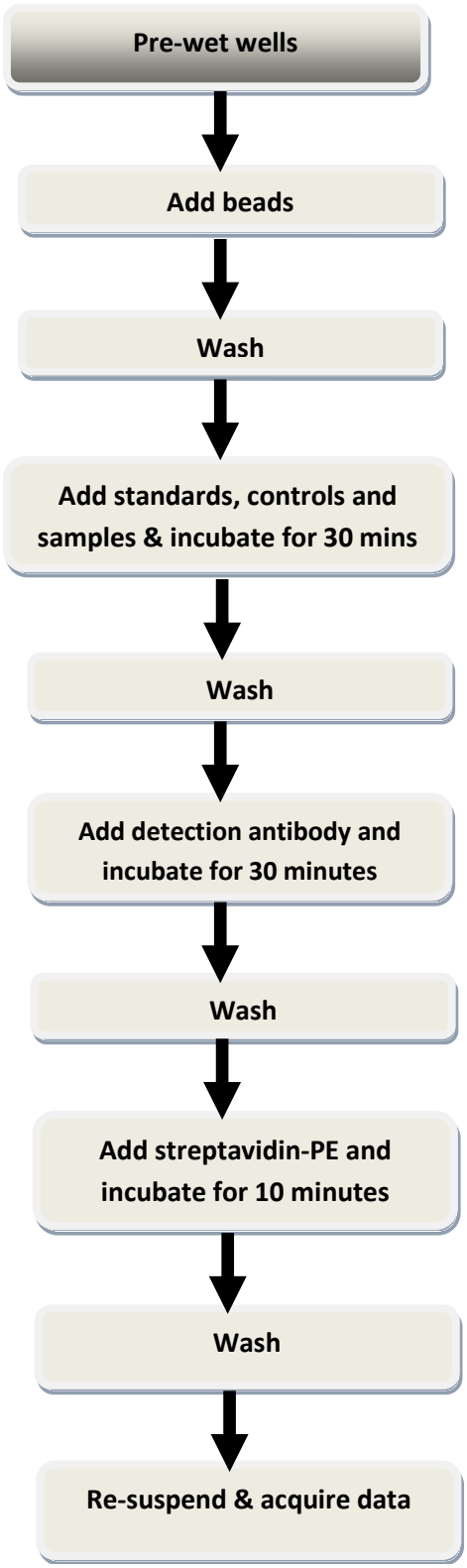


Figure 24: Bioplex system assay work flow (adapted from the instructor's manual)

6.12.4 Urinary Neopterin

Immune activation is associated with the release of neopterin[343-345], hence its widespread use as a marker of immune activation. It is elevated in infections such as TB[346], malaria[345] and HIV[347], cardiovascular diseases and autoimmune diseases such as rheumatoid arthritis and systemic lupus. In order to assess the effect of weekly chloroquine on the *plasmodium*-induced macrophageal activation, I assessed neopterin levels in urine samples at three time points – days 3, 15 and 30. These time points were chosen to capture the neopterin response during the different phases of the malaria infection – day 3 was to capture the acute phase, day 15 the convalescent phase and day 30 the post-infection phase. The urine samples were placed in a cold box containing ice packs immediately after collection and transported to Keneba MRC lab. Once in the lab, 2mls aliquot of the sample was placed in Eppendorf tubes and labelled with the subject's ID, date of the study, study day and type of specimen. The urine specimen was stored at -20°C until analysed. Urinary neopterin levels were measured using the enzyme-linked immunosorbent assay (ELISA) technique. Prior to the analysis, the method was first optimised. The optimisation was done by preparing serial dilutions of urine (1:1, 1:10, 1:100, 1:1000, 1:10,000) and measuring the neopterin concentration using the ELISA technique. The dilution with the fewest number of out of range values was taken to be the optimal dilution. This corresponded to a dilution of 1:100. The stored urine was allowed to thaw at room temperature and then diluted 1:100; the rest of the procedure was according to the instructions supplied by the manufacturers of the kit. The neopterin kit used was procured from BRAHMS Neopterin EIA (B.R.A.H.M.S, Germany) (Box 2).

Box 2: The BRAHMS Neopterin EIA (From the manufacturer's manual)

The B·R·A·H·M·S Neopterin EIA is a competitive enzyme immunoassay for the quantitative determination of neopterin in serum, plasma and urine using coated microtitre plates. The wells of the microtitre plate are coated with anti-neopterin antibodies (polyclonal, sheep). After addition of the enzyme conjugate (neopterin/alkaline phosphatase conjugate) to standards and control sera and to patient samples (serum, plasma or urine) the neopterin of the patient samples competes with the neopterin/enzyme conjugate for the binding sites of these antibodies, thus forming an immune complex bound to the solid phase (anti-neopterin antibody/neopterin or neopterin/enzyme conjugate). The subsequent intensive washing step ensures the complete removal of all unbound components. The addition of the 4-nitrophenyl phosphate substrate solution starts the enzyme reaction in which the alkaline phosphatase contained in the neopterin/enzyme conjugate catalyses the cleavage of the phosphate of 4-nitrophenyl phosphate, thus forming the yellow 4- nitrophenol. The enzymatic reaction is stopped by alkalinisation with sodium hydroxide. The intensity of the colour (measured in optical density OD) depends on the quantity of enzyme bound for a constant reaction time and consequently is inversely proportional to the neopterin concentration in the patient sample. Thus, high neopterin values correspond to a low optical density. The optical density is measured by means of a microtitre plate reader at an absorption maximum of 405 nm. The results are calculated by plotting a standard curve (optical density versus concentrations of neopterin standard), from which the neopterin concentrations in the patient samples can be read off directly.

6.13 Statistical analysis

6.13.1 Analysis sets

The data were first analysed according to the malaria season (i.e. 2007 and 2008), and then pooled. In 2007, there were four sub-groups:

1) Sub-group 1 – children treated initially with CQ-SP and subsequently received weekly chloroquine

2) Sub-group 2 - children initially treated with CQ-SP and subsequently received weekly placebo

3) Sub-group 3 - children treated initially with ACT and subsequently received weekly chloroquine

4) Sub-group 4 - children treated initially with ACT and subsequently received weekly placebo

The 2008 datasets contained only two groups – children randomised to either weekly chloroquine or weekly placebo. However, because of the small numbers at the two malaria seasons, the two datasets were pooled.

The analysis focused mainly on the children who participated in the weekly chloroquine/weekly placebo randomisation phase. Malaria incidence was presented separately for 2007 and 2008. Comparison of the effect on Hb of treating a child with either CQ-SP or ACT was only possible for 2007 data as CQ-SP was not used in 2008. Also, because cytokine was measured only in 2008, only the 2008 data were used for this analysis. For all the other analyses, pooled data were used. The data were presented by randomisation group (chloroquine/placebo). For all analyses on Hb change, day three Hb was used as the baseline while day 0 Hb was referred to as the Hb at recruitment since the Hb at day three determined which child was randomised to weekly chloroquine or weekly placebo.

Summary statistics were presented as means and standard deviations for continuous variables that are normally distributed, and counts and percentages for discrete variables. Neopterin and parasite count were not normally distributed and were log transformed ($\log_e$ for neopterin and $\log_{10}$ for parasite count). The geometric means and 95% confidence intervals were presented for log-transformed variables. Two sided student's t test was used to compare mean change in Hb at days 30 and 90 between weekly chloroquine and weekly placebo groups.

Analysis of mean Hb changes from day 15 to day 90 in the two randomisation arms suggested that by day 30, the Hb change had reached a plateau with little or no further change occurring. The Hb data from day 30 to day 90 were therefore pooled. This pooled Hb was referred to as the 'final Hb' and represents the combined Hb as a result of the intervention. A random effects model was used to further explore the differences in Hb response between the two randomisation arms. The random effects model is robust, and takes into account random differences across measurements and missing values. In building the model, the main comparators were the final Hb and the randomisation

groups, while controlling for the treatment groups (CQ-SP vs ACT), Hb at recruitment (day 0 Hb), Hb at day three, age in months, year of study, and the child's village. Similar analyses using the random effects model were conducted for the three markers of iron status measured in this study (MCV, MCH and ZnPP).

Linear regression was used to investigate the predictors of Hb change in the study population. First of all, simple linear regression was used to assess the association of each predictor variable with Hb. Thereafter, all variables with a P-value of 0.1 or less were included in a multiple linear regression model to assess confounding. The final regression model consisted of only the variables that maintained a significant association with Hb in the multiple regression model.

Two sided Student's t-test was used to compare mean change in Hb at days 7 to 90 between CQ-SP and ACT treatment arms in the placebo arms. The association was further explored using a random effects model with Hb and treatment as the comparators while controlling for Hb at recruitment (day 0 Hb), Hb at day three, age in months, year of study, and the child's village. In this model, Hb was pooled from day 15 to day 90 to get a single Hb. Student's t-test was also used to compare the mean change in Hb between children who were iron deficient and those who were not iron deficient.

Urinary neopterin concentrations were skewed and therefore log-transformed to convert it to normality. Neopterin response in the weekly chloroquine and placebo groups were compared at each of the observation time points using Student's t-test.

For cytokine data acquired from the whole blood culture supernatants, the net cytokine responses were derived by subtracting the cytokine levels in the unstimulated wells from the levels in the stimulated wells except the cytokine levels in response to PfSE where the net cytokine values were derived by subtracting the levels in the uRBC wells from the levels in the PfSE wells. Most of the net cytokine values were skewed and contained negative values (suggesting lower cytokine levels in the stimulated compared to the unstimulated wells); hence the data could not be transformed. Therefore non-parametric tests were used to analyse the data. Wilcoxon signed tests (two-sided) were used to

compare days three and 45 cytokine levels for each of weekly chloroquine and weekly placebo groups; while Mann Whitney U test was used to compare the cytokine responses at day 45 between the weekly chloroquine and weekly placebo arms. Comparisons of the day three responses in the two randomisation arms was not done because weekly supplementation commenced at study day 10 (i.e. one week after the day three sample was collected) and therefore did not reflect an effect of the intervention. All the analyses were first conducted at a 95% significance limit with a P-value of 0.05 being considered provisionally significant. Thereafter, a Bonferroni correction for multiple testing was conducted by multiplying the provisional P-value by 7 (the number of cytokines tested). Comparisons that retained a p-value of at 0.05 or less after Bonferroni correction were taken to be definitely significant. All analyses were carried out using STATA version 8 (StataCorp, College Station, TX, USA).

Outcome measures for the study

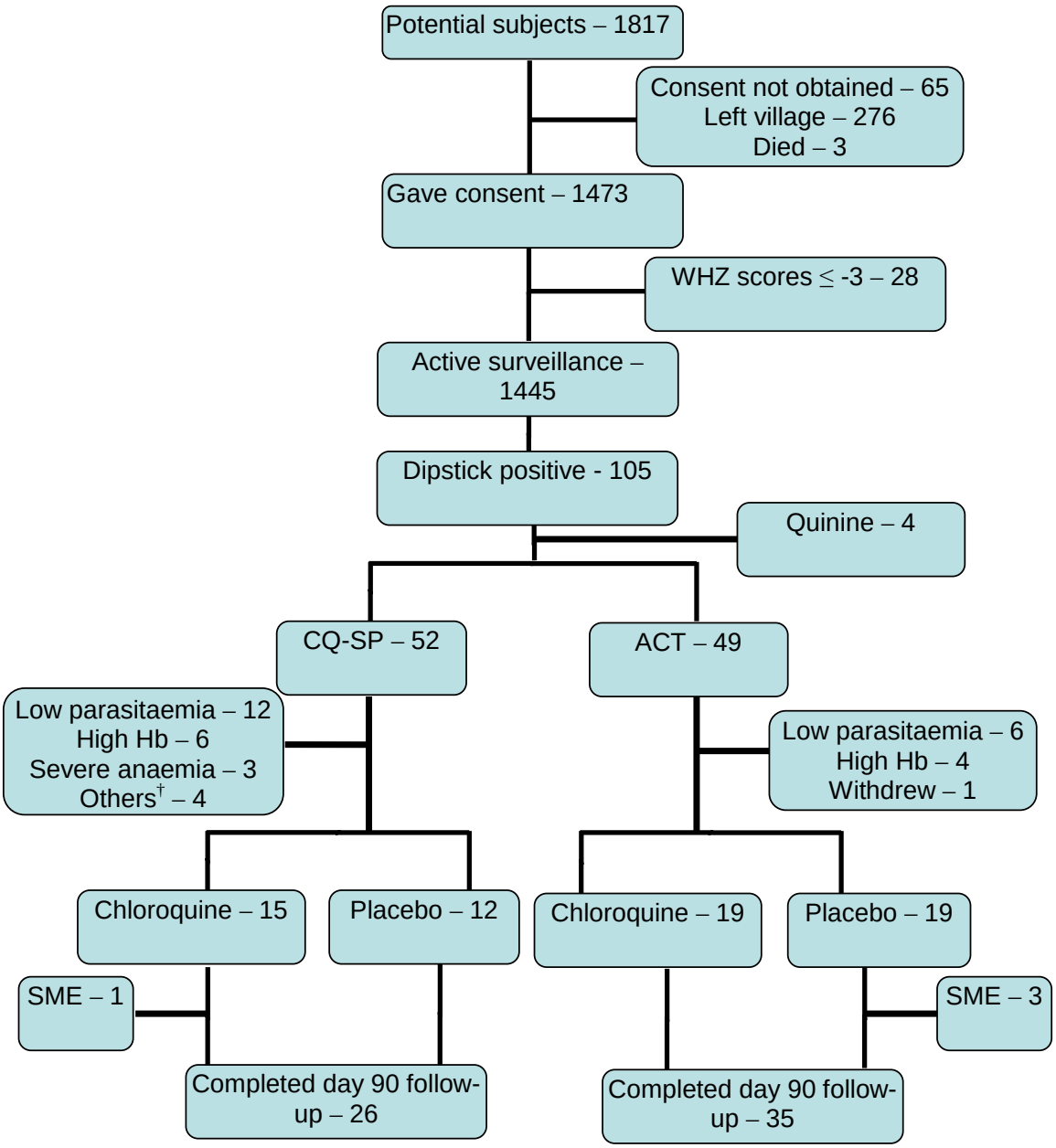
- **Primary Outcome Measure**
  - **Changes in Hb concentration from day 3 post treatment of malaria episode to day 90 in the weekly chloroquine and placebo arms.**

- **Secondary Outcome Measures**
  - **Curve of Hb change from day 3 to day 90 in the two placebo arms**
  - **Changes in markers of iron status, and erythropoietic response between day 3 and day 30, and between day 3 and day 90**
  - **Changes in measures of inflammation**
    - plasma ratios of pro and anti-inflammatory cytokines between day 3 and day 30, and between day 3 and day 90 – plus
    - urinary neopterin as a marker of macrophage activation
  - **Malaria parasitaemia using PCR to assess low-grade sub-microscopic parasitaemia**

7 Chapter 7 – Results

7.1 Recruitment and follow-up

The study was conducted over two malaria seasons. In 2007, 1817 children in 14 villages were identified through the Keneba MRC DSS database of which 1445 were eventually placed under malaria surveillance. Figures 25 & 26 describe the movement of the participants in and out of the study.



SME = second malaria episode; † 'Others' include early treatment failure (ETF) – 1; left village after day 3 – 1; refused bleeding – 1; and referred to the supplement centre – 1

Figure 25: CONSORT Flow Diagram describing participants' movements in 2007

In 2008, five of the 14 villages used in 2007 were dropped for logistic reasons as already explained above. In the remaining 9 villages, 1413 children were identified through the database, of which 1220 were placed under malaria surveillance. A total of 96 children were randomised to weekly chloroquine and weekly placebo supplementation arms.

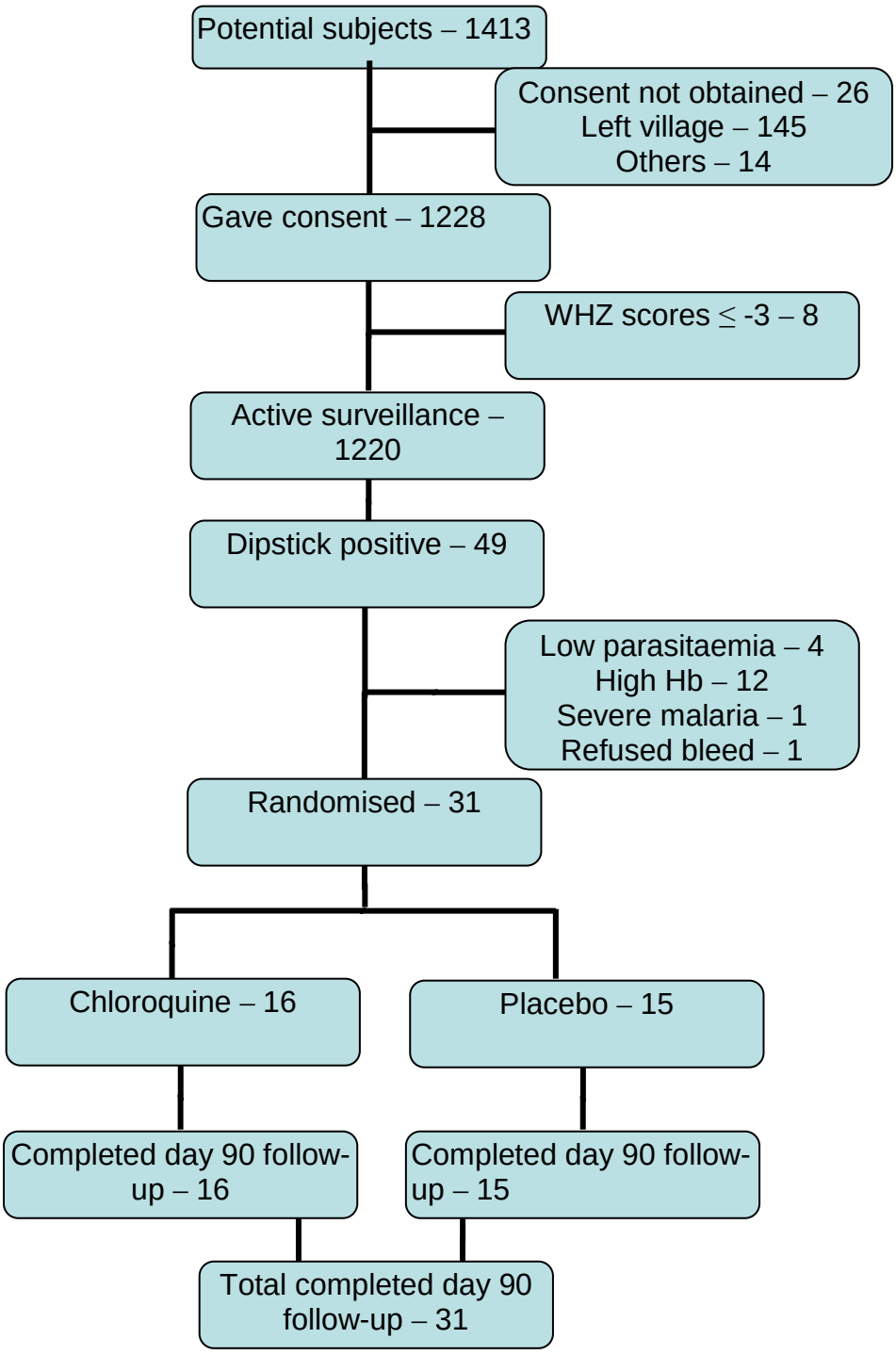


Figure 26: CONSORT Flow Diagram describing participants' movements in 2008

7.2 Baseline Characteristics of the study population

7.2.1 General characteristics of the study population

In 2007, 746 (51.4%) of the participants were males and 705 (48.6%) were females; in 2008, there were 616 (50.5%) males and 604 (49.5%) females. Table 8 describes the characteristics of all the children placed under surveillance for malaria. The incidence of malaria fell from 7.3% in 2007 to just 4% in 2008. One hundred and fifty-four children (5.8%) were dipstick positive over the period (105 in 2007 and 49 in 2008); 96 were subsequently randomised to receive either weekly chloroquine or weekly placebo (65 in 2007 and 31 in 2008). All the 96 children randomised were included in this analysis.

Table 8: Baseline characteristics of all participants under surveillance

	2007	2008	Combined
N (%)	1445	1220	2665
Age in months (SD)	42.2 (17.2)	40.7 (17.7)	41.5 (17.5)
Weight-for-Height Z-scores (SD)	-1.06 (0.84)	-1.02 (0.85)	-1.04 (0.84)
Weight-for-Age Z-scores (SD)	-1.48 (0.97)	-1.50 (0.94)	-1.49 (0.95)
Height-for-Age Z-scores (SD)	-1.06 (1.21)	-1.17 (1.13)	-1.11 (1.17)
Body Mass Index (SD)	14.6 (1.3)	14.7 (1.4)	14.6 (1.4)
Dipstick positive			
No (%)	1340 (92.7)	1171 (96.0)	2511 (94.2)
Yes (%)	105 (7.3)	49 (4.0)	154 (5.8)

In the two malaria seasons, there were no significant differences in age, WHZ scores and BMI of children that tested dipstick positive and those who did not (Tables 9 & 10).

Table 9: Comparison of characteristics of 2007 participants that were dipstick positive and those who were not; the figures reported are means (standard deviations)

	Dipstick positive		Total	P-value
	No	Yes		
N (%)	1340 (92.7)	105 (7.3)	1445	
Age in months (SD)	42.0 (17.2)	44.7 (17.8)	42.2 (17.2)	0.1
Weight-for-Height Z-scores (SD)	-1.08 (0.83)	-0.93 (0.86)	-1.06 (0.84)	0.09
Weight-for-Age Z-scores (SD)	-1.50 (0.95)	-1.24 (1.14)	-1.48 (0.97)	0.007
Height-for-Age Z-scores (SD)	-1.08 (1.17)	-0.89 (1.57)	-1.06 (1.21)	0.1
Body Mass Index (SD)	14.6 (1.3)	14.7 (1.4)	14.6 (1.3)	0.5

Table 10: Comparison of characteristics of 2008 participants that were dipstick positive and those who were not; the figures reported are mean (standard deviations)

	Dipstick positive		Total	P-value
	No	Yes		
N (%)	1171 (96.0)	49 (4.0)	1220	
Age in months (SD)	40.7 (17.7)	39.1 (18.3)	40.6 (17.7)	0.5
Weight-for-Height Z-scores (SD)	-1.01 (0.85)	-1.13 (0.69)	-1.02 (0.85)	0.3
Weight-for-Age Z-scores (SD)	-1.49 (0.95)	-1.76 (0.72)	-1.50 (0.94)	0.05
Height-for-Age Z-scores (SD)	-1.16 (1.14)	-1.45 (0.84)	-1.17 (1.13)	0.07
Body Mass Index (SD)	14.7 (1.4)	14.5 (1.1)	14.7 (1.4)	0.2

7.2.2 Characteristics of participants treated with chloroquine-sulphadoxine-pyrimethamine (CQ-SP) and those treated with co-artemether (ACT)

In 2007, the subjects were initially randomised to receive either the standard treatment for malaria as at that time (CQ-SP) or ACT (which was increasingly becoming a popular first line antimalarial drug). This was an open randomisation in which participants were sequentially allocated to either treatment. Table 11 compares the baseline characteristics of the children randomised to the standard treatment and those randomised to the ACT treatment. There was no significant difference between the two groups in all the parameters compared. There was only one early treatment failure and that occurred in the CQ-SP arm.

Table 11: Comparison of characteristics of participants who were treated with ACT versus those treated with CQ-SP in 2007

	ACT	CQ-SP	Total	P-value
N (%)	48 (48)	52 (52)	100	
Age in months (SD)	44.8 (19.1)	45.6 (16.3)	45.2 (17.7)	0.8
Height-for-Age Z-scores	-0.99 (1.11)	-0.91 (1.25)	-0.95 (1.18)	0.7
Weight-for-Age Z-scores	-1.35 (0.96)	-1.23 (1.00)	-1.29 (0.98)	0.5
WHZ (SD)	-0.96 (0.82)	-0.85 (0.95)	-0.91 (0.89)	0.5
BMI (SD)	14.7 (1.5)	14.8 (1.6)	14.7 (1.5)	0.6
Initial parasitaemia[†]	23998 (13806, 41714)	22633 (12780, 40083)	23313 (15785, 34432)	0.8
Hb at day 0 (SD)	101 (15)	102 (17)	102 (16)	0.7
Hb at day 3 (SD)	95 (12)	96 (18)	95 (16)	0.6

[†]Geometric mean reported

7.2.3 Characteristics of participants randomised to weekly chloroquine and weekly placebo

This part of the study was double blind with allocation made sequentially. The similarity of participants in either arm gives an indication of the effectiveness of the randomisation. Tables 12 - 14 compare the baseline characteristics of participants who were randomised to weekly chloroquine and weekly placebo. The results indicate that there was no significant difference between the two groups.

Table 12: Comparison of the baseline characteristics of participants randomised to weekly CQ vs weekly placebo in 2007

	Chloroquine	Placebo	Total	P-value
N (%)	34 (52.3)	31 (47.7)	65	
Age in months (SD)	45.9 (17.7)	41.7 (19.4)	43.9 (18.5)	0.4
Height-for-Age Z-scores	-1.00 (0.84)	-0.79 (1.38)	-0.90 (1.13)	0.5
Weight-for-Age Z-scores	-1.23 (0.89)	-1.25 (0.98)	-1.24 (0.93)	0.9
WHZ (SD)	-0.79 (0.92)	-0.96 (0.83)	-0.87 (0.88)	0.4
BMI (SD)	14.9 (1.5)	14.7 (1.4)	14.8 (1.5)	0.6
Day 0 Hb (g/L, SD)	103 (14)	97 (14)	100 (14)	0.1
Day 3 Hb (g/L, SD)	95 (14)	92 (10)	93 (12)	0.3
Treatment				
ACT (%)	19 (55.9)	19 (61.3)	38 (58.5)	0.7
CQ-SP (%)	15 (44.1)	12 (38.7)	27 (41.5)	
Parasitaemia (per µL; 95% CI)[†]	49,357 (29,093; 83,735	27,576 (16,890; 45,021)	37,391 (26,097; 53,574)	0.1
Iron deficiency				
No (%)	22 (64.7)	15 (48.4)	37 (56.9)	0.2
Yes (%)	12 (35.3)	16 (51.6)	28 (43.1)	

[†]Geometric mean reported

Table 13: Comparison of the baseline characteristics of participants randomised to weekly CQ vs weekly placebo in 2008

	Chloroquine	Placebo	Combined	P-value
N (%)	16 (51.6)	15 (48.4)	31	
Age in months (SD)	42.9 (17.9)	42.5 (13.6)	42.7 (15.7)	0.9
Height-for-Age Z-scores	-1.58 (0.96)	-1.25 (0.64)	-1.42 (0.82)	0.3
Weight-for-Age Z-scores	-1.90 (0.93)	-1.55 (0.53)	-1.73 (0.77)	0.2
WHZ (SD)	-1.24 (0.80)	-0.96 (0.72)	-1.11 (0.76)	0.3
BMI (SD)	14.4 (1.0)	14.7 (1.3)	14.6 (1.2)	0.4
Day 0 Hb (g/L, SD)	106 (16)	109 (13)	108 (14)	0.5
Day 3 Hb (g/L, SD)	94 (9)	98 (9)	96 (9)	0.2
Parasitaemia (per µL; 95% CI)[†]	32,763 (15,757, 68,122)	35,125 (16,278, 75,795)	33,885 (20,589, 55,768)	0.9
Iron deficiency				
No (%)	5 (31.2)	8 (53.3)	13 (41.9)	0.2
Yes (%)	11 (68.8)	7 (46.7)	18 (58.1)	

[†]Geometric mean reported

Table 14: Baseline characteristics of children randomised to weekly chloroquine and weekly placebo in 2007 and 2008, pooled

Parameter[†]	Chloroquine group	Placebo group	Combined
Sample size (%)	50 (52.1)	46 (47.9)	96
Sex			
Female (%)	22 (44)	22 (47.8)	44 (45.8)
Male (%)	28 (56)	24 (52.2)	52 (54.2)
Age in months, mean (SD)	41.9 (18.1)	38.7 (17.8)	40.3 (17.9)
Height-for-Age Z-scores (SD)	-1.19 (0.91)	-0.94 (1.20)	-1.07 (1.06)
Weight-for-Age Z-scores (SD)	-1.44 (0.95)	-1.35 (0.87)	-1.40 (0.90)
Weight-for-Height z scores (SD)	-0.94 (0.90)	-0.96 (0.79)	-0.95 (0.85)
BMI (SD)	14.7 (1.4)	14.7 (1.3)	14.7 (1.4)
Day 0 parasitaemia, geometric mean (95% CI)	47783 (31617, 71120)	32496 (21910, 47507)	39792 (29995, 52403)
Day 0 Hb, g/L (SD)	103.9 (14.2)	101.5 (14.8)	102.7 (14.4)
Day 3 Hb, g/L (SD)	93.4 (9.6)	93.7 (10.2)	93.6 (9.8)
Presence of iron deficiency			
No (%)	27 (54)	23 (50)	50 (52.1)
Yes (%)	23 (46)	23 (50)	46 (47.9)

[†]Except otherwise stated, numeric variables were reported as means (SD) while discrete variables were reported as counts (%)

7.2.4 Iron deficiency

As discussed in chapter six, we used three parameters to define iron deficiency: MCV, MCH and ZnPP. The cut off values for the different parameters employed were : 61µmol/mol of Hb for ZnPP[129], 73fl for MCV[330] and 25pg for MCH[330]. Combining these parameters has been shown to increase their sensitivity and specificity to identify iron deficiency. Using this modality, the prevalence of iron deficiency among the study population was 43.1% (28/65) in 2007 and 58.1% (18/31) in 2008. The combined prevalence for the two malaria seasons was 47.9% (46/96). There was no significant difference in the prevalence of iron deficiency between children in the two randomisation groups. Children with WAZ score <-2 had higher prevalence of iron deficiency compared with children with WAZ score ≥-2 (78.6% vs 58.8%; p = 0.001). Similarly, children with WHZ score <-2 had higher prevalence of iron deficiency compared with children with WHZ score ≥-2 (100% vs 60%; p < 0.001). However, there was no significant difference in the prevalence of iron deficiency between children with a HAZ score <-2 and children with HAZ score ≥-2 (71.4% vs 62.1%; p = 0.2). Iron deficient children had a higher BMI (14.9 ± 1.5) compared with children who were not iron deficient (14.6 ± 1.3; p = 0.03). Children with iron deficiency were also significantly younger (mean age (SD) = 38.0 ± 18.7 months) than children without iron deficiency (mean age (SD) = 54.2 ± 12.4 months). Children aged below three years had a higher prevalence of iron deficiency (96.0%) than children aged three years and above (42.5%, p <0.001). There was no difference in the prevalence of iron deficiency between the village groups. The clinical correlates of iron deficiency are described in section 7.7.2.

7.3 Haemoglobin change from day 3 to day 90

The primary outcome of this study was to determine the difference in Hb change at days 30 and 90 in children who received weekly chloroquine compared with those who received weekly placebo. This measure is to assess the effect of weekly chloroquine on haemoglobin recovery after complete clearance of malaria parasitaemia. Haemoglobin change was determined by subtracting the Hb at each of the observation points from the Hb at day three. Table 15 compares the mean change in Hb in the weekly chloroquine group with the weekly placebo group for 2007 and 2008 data separately and also the pooled data. The results show that there were no significant differences in the Hb change between the two groups.

Table 15: Change in HB from day 3 to day 30; and from day 3 to day 90

		Chloroquine	Placebo	Mean difference, 95%CI	P-value
2007	Hb change from day 3 to day 30, g/L (sd)	9.6 (12.3)	9.0 (12.6)	0.6 (-5.8, 7.1)	0.8
	Hb change from day 3 to day 90, g/L (sd)	11.6 (13.5)	8.9 (14.5)	2.6 (-4.8, 10.1)	0.5
2008	Hb change from day 3 to day 30, g/L (sd)	2.5 (10.4)	7.8 (9.9)	-5.4 (13.3, 2.5)	0.2
	Hb change from day 3 to day 90, g/L (sd)	3.7 (10.4)	5.3 (17.2)	-1.7 (12.0, 8.7)	0.7
Pooled	Hb change from day 3 to day 30, g/L (sd)	7.4 (12.1)	8.6 (11.7)	-1.2 (-6.2, 3.9)	0.6
	Hb change from day 3 to day 90, g/L (sd)	10.0 (11.7)	7.3 (13.3)	2.7 (-2.7, 8.1)	0.7

Figures 27 to 29 are bar charts comparing the changes in Hb at the different time points between the two randomisation arms. The figures suggest that by day 30, the Hb change had reached a plateau with little or no further change in Hb occurring.

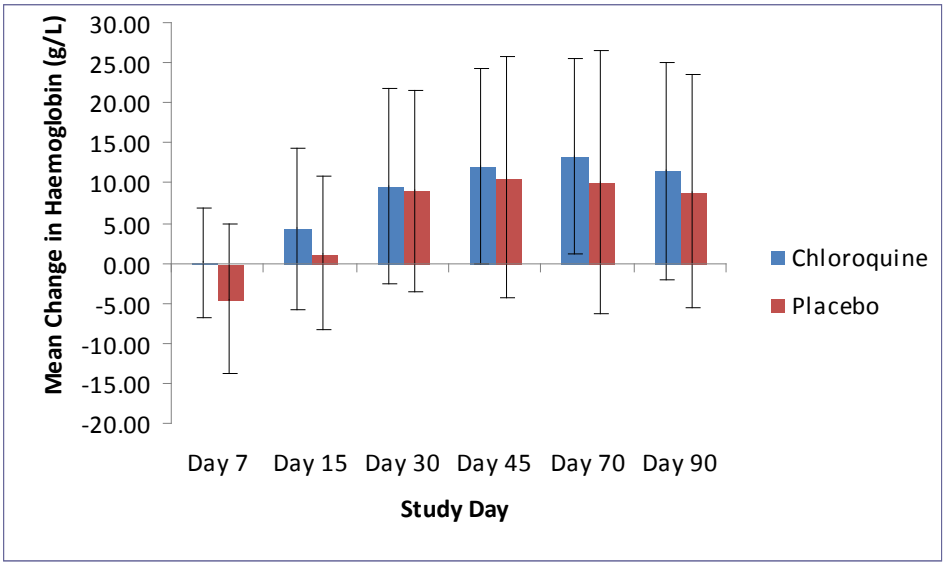


Figure 27: Bar chart showing the mean Hb change in 2007. Error Bars represent Standard Deviations

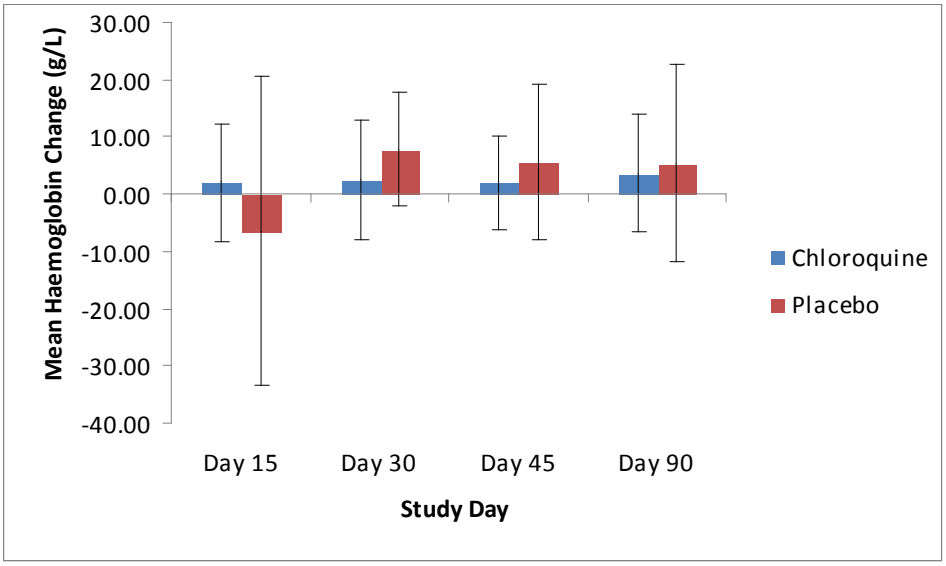


Figure 28: Bar chart showing the mean Hb change in 2008. Error Bars represent Standard Deviations

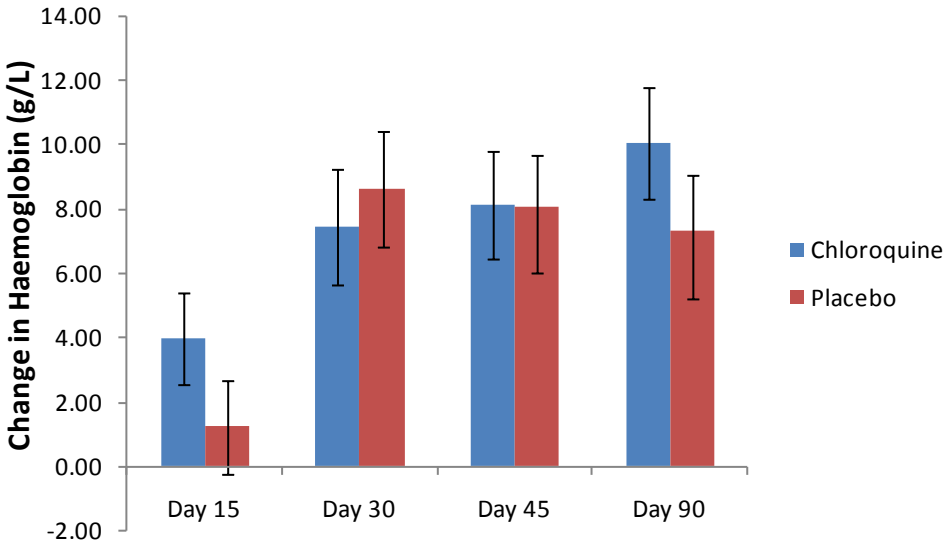


Figure 29: Bar chart comparing the change in Hb between children randomised to weekly chloroquine and those randomised to weekly placebo (pooled data). Error Bars represent Standard Deviations.

To further explore the differences in Hb responses between the weekly chloroquine and placebo arms, we used random effects modelling which pools the Hb data across the plateau phase (days 30, 45 and 90). In the random effects model, there is a single Hb value which I called the 'final Hb' in place of three Hb values (days 30, 45 and 90 Hb levels).

Model 1: Random effects model exploring the relationship between Hb and treatment and randomisation groups (Pooled data)

¥i.random _Irandom_1-2 (naturally coded; _Irandom_1 omitted)
¥i.treat _Itreat_1-2 (naturally coded; _Itreat_1 omitted)
¥i.vill _Ivill_1-4 (naturally coded; _Ivill_1 omitted)
¥i.year _Iyear_2007-2008 (naturally coded; _Iyear_2007 omitted)

Random-effects GLS regression				Number of obs =		344
Group variable (i): idno				Number of groups =		94
R-sq:	within	0.0000		Obs per group: min =		1
	between	0.6625		avg =		3.7
	overall	0.5178		max =		4
Random effects u_i ~ Gaussian					Wald chi2(9) =	170.81
corr(u_i, X) = 0 (assumed)					Prob > chi2 =	0.0000
Final Hb	Coef.	Std. Err.	z	P>z	[95% Conf. Interval]	
Day 3 Hb	0.1795366	0.1152988	1.56	0.119	-0.0464449	0.4055181
Day 0 Hb	0.403079	0.0835638	4.82	0.000	0.239297	0.5668611
_Irandom_2	1.222722	1.480054	0.83	0.409	-1.678131	4.123574
_Itreat_2	-0.5381094	1.825875	-0.29	0.768	-4.116758	3.040539
_Ivill_2	2.500234	4.214512	0.59	0.553	-5.760058	10.76053
_Ivill_3	0.354331	2.022824	0.18	0.861	-3.610332	4.318994
_Ivill_4	3.826106	1.693215	2.26	0.024	0.5074663	7.144745
Age in months	0.2148899	0.0465366	4.62	0.000	0.1236799	0.3060998
_Iyear_2008	-2.422581	1.93317	-1.25	0.21	-6.211524	1.366362
_cons	32.88111	7.267618	4.52	0.000	18.63684	47.12538
sigma_u	5.755255					
sigma_e	7.6315902					
rho	0.36253791	(fraction of variance due to u_i)				

¥These are dummy variables automatically created by STATA. The dummy corresponding to the smallest value of the variable is dropped by default. "random" = randomisation group (weekly CQ or weekly placebo); "treat" = initial antimalarial treatment received (CQ-SP or ACT); "vill" = child's village group (village 1 = Jiffarong, village 2 = Karantaba, village 3 = Keneba, village 4 = Sankandi)

In building the random effects model I first evaluated the relationship between the randomisation group (weekly chloroquine versus weekly placebo) and the final Hb, controlling for the Hb at recruitment (day 0), Hb at baseline (day 3), age in months, year of study, initial treatment received, and the child's village group. This model showed that giving weekly chloroquine did not have any effect on the final Hb (Model 1). The model also showed that the intial antimalarial given to the child did not have any effects on the final Hb after controlling for

the above variables. Adding parasitaemia levels at recruitment and at follow-up, and presence of iron deficiency to the model did not change this relationship.

A post-hoc analysis of the effect size based on the actual sample size was conducted using the formula:

$d = t(n_1 + n_2) / [\sqrt{}(df)\sqrt{}(n_1 n_2)]$

where

d is Cohen's *d* or the difference between the mean Hb change in the chloroquine and placebo arms divided by the pooled standard deviation

t is the t statistic for the t test of the mean Hb change for the chloroquine and placebo groups

n1 is the sample size for chloroquine

n2 is the sample size for placebo, and

df is the degrees of freedom for the t test.

The pooled standard deviation is the root mean square of the standard deviations of the chloroquine and placebo groups:

$\sigma_{pooled} = \sqrt{}[(\sigma_1^2 + \sigma_2^2)/2]$

Where

σ_1 is the standard deviation for chloroquine group, and

σ_2 is the standard deviation for placebo group

This gave a *d* value of 0.05, a very small effect size by Cohen's standards (http://www2.jura.uni-hamburg.de/instkrim/kriminologie/Mitarbeiter/Enzmann/Lehre/StatIIKrim/EffectSizeBecker.pdf, Accessed 28/01/2010).

7.4 Changes in markers of iron status

Mean corpuscular volume (MCV), mean cell haemoglobin (MCH) and zinc protoporphyrin (ZnPP) were used as markers of iron status. The changes in these parameters in relation to treatment and randomisation groups were explored. The results revealed that neither randomisation group (Table 16) nor initial antimalarial treatment given had any significant association with any of the markers of iron status.

Table 16: Changes in markers of iron status from day 3 to day 30; and from day 3 to day 90

	Chloroquine	Placebo	Mean difference, 95%CI	P-value
Change in MCV from day 3 to day 30, g/L (sd)	2.07 (3.66)	1.46 (3.00)	0.61 (-0.82, 2.04)	0.4
Change in MCV from day 3 to day 90, g/L (sd)	-1.29 (4.08)	-1.16 (4.57)	-0.13 (-1.98, 1.71)	0.9
Change in MCH from day 3 to day 30, g/L (sd)	-0.11 (1.44)	-0.01 (1.13)	-0.10 (-0.66, 0.45)	0.7
Change in MCH from day 3 to day 90, g/L (sd)	-1.25 (1.81)	-0.96 (1.73)	-0.29 (-1.05, 0.47)	0.5
Change in ZnPP from day 3 to day 30, g/L (sd)[‡]	2 (-14, 12)	14 (-1, 32)		0.06
Change in ZnPP from day 3 to day 90, g/L (sd)	7 (-1, 32)	19 (-4, 47)		0.4

[‡]Data were skewed & contained many negative values making transformation not possible; therefore Mann-Whitney U test for independent samples was used to test the difference. Figures reported are median values and interquartile ranges.

The pattern of reticulocyte response was examined as a marker of bone marrow response to the intervention. Figure 31 is a graph showing the reticulocyte response across the observation period. The reticulocyte response peaked at day 15 and returned to baseline values by 30. It remained at this level until day 90. This pattern was similar to that seen in the weekly placebo group.

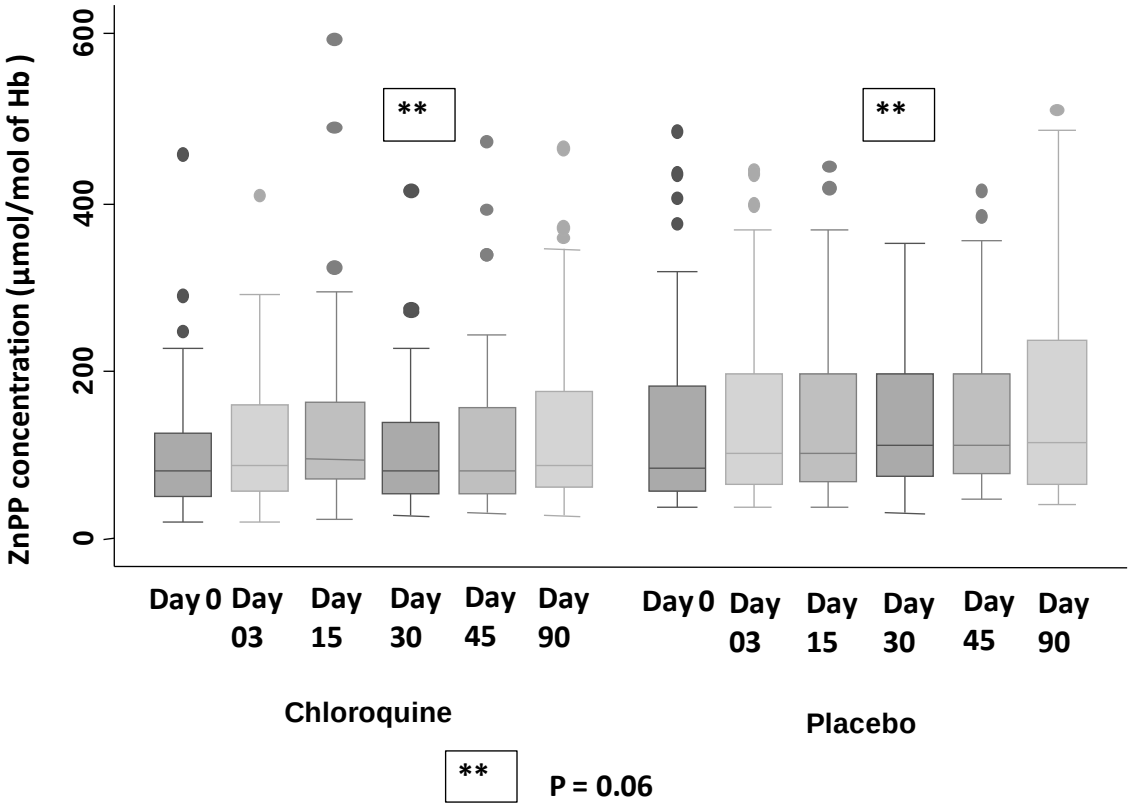


Figure 30: Box plot comparing erythrocyte zinc protoporphyrin between the different days in the weekly chloroquine and weekly placebo groups.

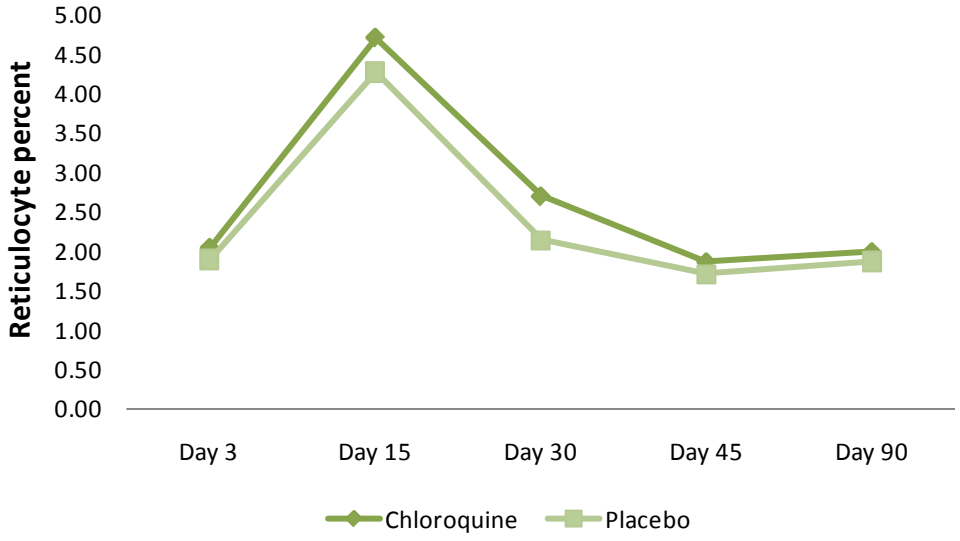


Figure 31: Reticulocyte percentage in chloroquine and placebo groups

Although at each observation point, the reticulocyte response in the chloroquine group was better than in the weekly placebo group, this did not achieve any statistical significance (Table 17). The difference between the two groups was more noticeable at day 30.

Table 17: Reticulocyte percent at the different observation days in children randomised to weekly chloroquine vs children randomised to weekly placebo

	Geometric Mean (95% conf. Interval)		
Study Day	Chloroquine	Placebo	P-value
3	1.71 (1.45, 2.01)	1.56 (1.31, 1.85)	0.4
15	3.81 (3.14, 4.63)	3.58 (2.93, 4.36)	0.6
30	2.23 (1.86, 2.67)	1.94 (1.70, 2.22)	0.2
45	1.70 (1.50, 1.93)	1.60 (1.44, 1.78)	0.5
90	1.69 (1.46, 1.96)	1.64 (1.41, 1.90)	0.7

7.5 Comparison of Hb response in participants given therapeutic doses of chloroquine-sulphadoxine-pyrimethamine (CQ-SP) and those given therapeutic doses of co-artemether (ACT)

One of the secondary outcome measures of this study was to examine the curve of haemoglobin change from day three to day 90 in the placebo arms as a measure of the effect of CQ-SP vs. ACT treatment on macrophageal iron loading and release in acute clinical malaria. This analysis was only possible in the 2007 participants. In 2007, the subjects were initially randomised to receive either the standard treatment for malaria as at that time (CQ-SP) or ACT. This was an open randomisation in which participants were sequentially allocated to either treatment. But because of the change in malaria treatment policy in The Gambia, this phase of the study was dropped, and all the children treated with ACT. Figure 32 is a bar chart of Hb change from day three to day 90 in the placebo arm stratified by the initial antimalarial treatment received. The figure suggests that Hb recovery was better in children treated with CQ-SP than those treated with ACT. Although the confidence intervals of the mean difference (Table 18) indicate that this difference was not statistically significant, the enhanced haematological response to CQ-SP compared with ACT is interesting. It is important to note that the dose of chloroquine used in the treatment of malaria (30mg/kg over three days) was higher than the recommended anti-inflammatory dose. It is likely that with a larger sample size, this difference might have achieved significance.

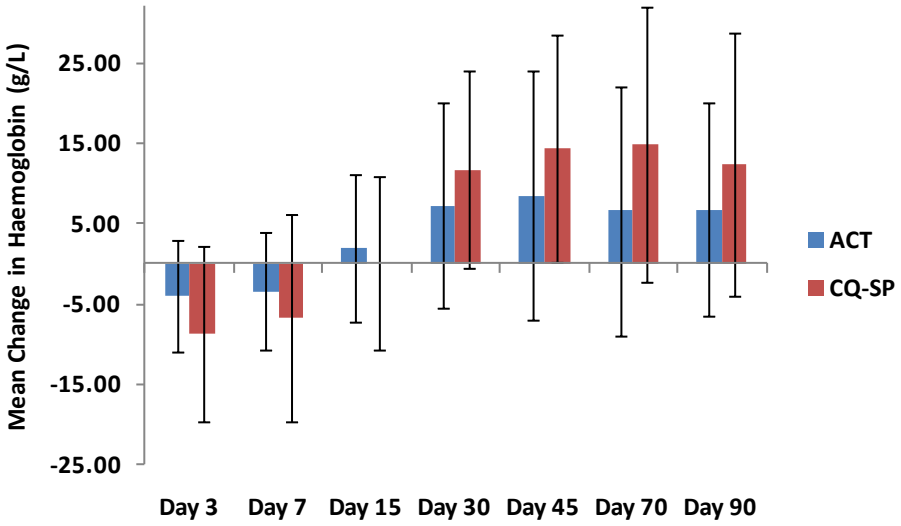


Figure 32: Hb change in the two placebo arms in 2007 comparing the effect on Hb recovery of treating malaria with either chloroquine-sulphadoxine-pyrimethamine (CQ-SP) or co-artemether (ACT)

Table 18: Change in Hb in children randomised to the placebo group comparing children who were initially treated with chloroquine-sulphadoxine-pyrimethamine (CQ-SP) and those treated with co-artemether (ACT)

Observation Day	ACT (n=19); mean (sd)	CQ-SP (n=12); mean (sd)	Mean Difference (95%CI)	P-value
Day 3	-3.9 (7.0)	-8.8 (10.9)	4.8 (-1.9, 11.5)	0.2
Day 7	-3.4 (7.4)	-6.7 (12.8)	3.3 (-5.6, 12.2)	0.4
Day 15	1.9 (9.2)	0 (10.8)	1.9 (-6.3, 10.1)	0.6
Day 30	7.2 (12.8)	11.7 (12.3)	-4.5 (-14.5, 5.6)	0.4
Day 45	8.4 (15.5)	14.4 (14.0)	-5.9 (-17.7, 5.9)	0.3
Day 70	6.6 (15.5)	14.8 (17.1)	-8.1 (-20.9, 4.6)	0.2
Day 90	6.8 (13.3)	12.4 (16.4)	-5.7 (-17.8, 6.5)	0.3

We explored this further in a random effects model, pooling Hb concentrations at days 15 to 90, controlling for age (in months), Hb at recruitment (day 0) and village (Model 2). The output still suggested that there was no difference in the Hb response in children treated with CQ-SP compared with those treated with ACT. In addition, there was no difference in the day 3 urinary neopterin between children treated with CQ-SP and those treated with ACT (geometric mean [95%CI]: 81.7µmol/L [52.7µmol/L, 126.7µmol/L] vs. 77.1µmol/L [55.4µmol/L, 107.3µmol/L], p = 0.8).

Model 2: Random effects model exploring the effect of treating a child with CQ-SP versus ACT on the final Hb among children randomised to receive weekly placebo in 2007

. xi: xtreg hgb i.village i.treat hb_recruit agem if random==2, i(idno)
[¥]i.village _Ivillage_1-4 (naturally coded; _Ivillage_1 omitted)
[¥]i i.treat _Itreat_1-2 (naturally coded; _Itreat_1 omitted)

Random-effects GLS regression				Number of obs =		154
Group variable (i): idno				Number of groups =		30
R-sq:	within	0.0000		Obs per group: min =		4
	between	0.7223		avg =		5.1
	overall	0.4884		max =		6
Random effects u_i ~ Gaussian					Wald chi2(9) =	60.24
corr(u_i, X) = 0 (assumed)					Prob > chi2 =	0.0000
Final Hb	Coefficients	Std. Err.	z	P>z	[95% Conf. Interval]	
_Ivillage_2	1.935326	8.36649	0.23	0.817	-14.4627	18.33334
_Ivillage_3	-2.16578	4.99549	-0.43	0.665	-11.9568	7.625198
_Ivillage_4	3.391225	3.148127	1.08	0.281	-2.77899	9.56144
_Itreat_2	2.260308	3.119608	0.72	0.469	-3.85401	8.374627
Day 0 Hb	0.466858	0.14638	3.19	0.001	0.179959	0.753758
Age in months	0.214813	0.097358	2.21	0.027	0.023995	0.405631
_cons	41.64874	12.49236	3.33	0.001	17.16417	66.13331
sigma_u	6.253417					
sigma_e	9.995752					
rho	0.281291	(fraction of variance due to u_i)				

[¥]These are dummy variables automatically created by STATA. The dummy corresponding to the smallest value of the variable is dropped by default. "treat" = initial antimalarial treatment received (CQ-SP or ACT); "vill" = child's village group (village 1 = Jiffarong, village 2 = Karantaba, village 3 = Keneba, village 4 = Sankandi)

7.6 Changes in measures of inflammation between day 3 and day 90

Neopterin and plasma cytokines were used as markers of inflammation.

7.6.1 Neopterin

The procedure for measuring neopterin was first optimised before the analysis. Neopterin was first assayed in undiluted urine and in serially diluted urine. The readings were compared and the reading that gave the least out-of range values

was taken as the optimal dilution for conducting the assay. This corresponded to a dilution of 1:100. This was then used for the rest of the analysis.

Figure 33 is a box plot comparing urinary neopterin levels in the weekly chloroquine and placebo groups. As expected, the concentrations were very high during the acute phase of the malaria infection in both groups. But by day 15, the neopterin concentrations had fallen dramatically to very low levels in both groups. There was no difference in the neopterin concentration between the two groups at each of the observation points (Table 19). One obvious finding from the box plot was that the gap between measurements of the neopterin was too wide because by day 15, macrophageal activation has subsided. In future studies, such measurements might need to be conducted within the first week of recruitment. There was a significant correlation between the presence of submicroscopic parasitaemia at day 30 and neopterin levels at day 30 (r=0.3, p=0.002) (see section 7.7.3).

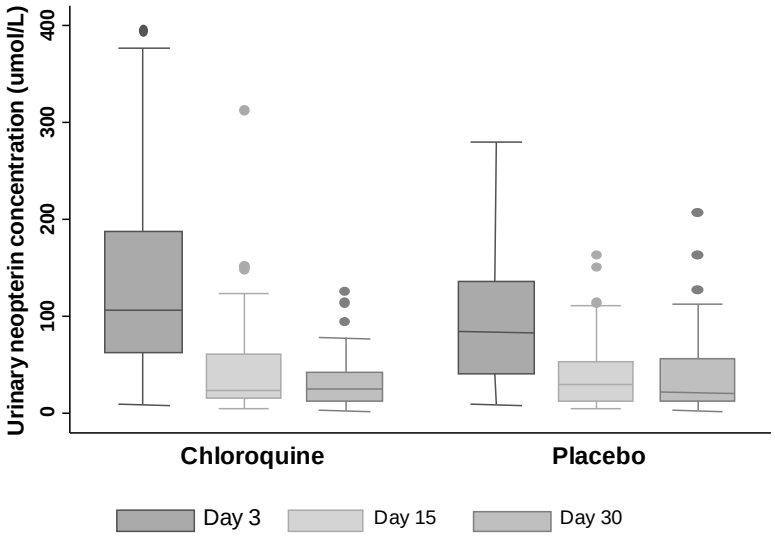


Figure 33: Urinary neopterin concentrations on days 3, 15 and 30 (The horizontal line inside the box represents the median neopterin concentration; the upper and lower horizontal lines of the box represent the 75th and 25th percentiles respectively; the bars represent the range of the neopterin concentrations, while the dots represent values more than 1.5 times the interquartile range (outliers))

Table 19: Comparison of urinary neopterin concentration between children on weekly chloroquine and those on weekly placebo

Study day	Chloroquine	Placebo	Mean difference, 95%CI	P-Value
Day 3 Neopterin: Geometric mean (95% CI)	92.1 (69.6, 121.8)	73.3 (55.3, 97.2)	1.3 (0.8, 1.9)	0.3
Day 15 Neopterin: Geometric mean (95% CI)	29.8 (22.3, 39.9)	27.8 (20.2, 38.3)	1.1 (0.7, 1.6)	0.7
Day 30 Neopterin: Geometric mean (95% CI)	25.3 (19.6, 32.6)	25.1 (18.1, 34.9)	1.0 (0.7, 1.5)	1.0

7.6.2 Plasma cytokines

7.6.2.1 Optimisation of the methods

A preliminary analysis of the plasma samples collected in 2007 was conducted using the Bio-Plex system as already described in section 6.11.3.2. The cytokine concentrations in the plasma samples were however very low with several out of range values below the detectable range for most of the cytokines particularly IL-10, IL-6, TNF-α and IL-12. It was therefore decided not to analyse the rest of the samples. To improve the cytokine yield, stimulated whole blood culture was used.

To analyse the cytokine response in the supernatants of the whole blood culture, the net cytokine yields of the overnight blood culture was first compared with the day 3 culture in the first six donors. The findings showed that the cytokine response for most of the conditions peaked at day 1 (i.e. after the overnight culture). In addition, the background cytokine values at day 3 were high. It was therefore decided to concentrate on the overnight (day 1) culture samples (Figure 34).

Each cytokine assayed was analysed for potential usefulness in providing information relevant to the hypothesis being tested. Based on this, IL-12 was dropped, and IL-13 and IL-17 were added. Thus it was decided to concentrate on the following cytokines: IL-6, IL-10, IL-13, IL-17, IFN-γ, TNF-α and MIF. Finally, the responsiveness of various antigens was assessed. Based on this criterion, AMA-1 and MSP-1 were dropped because of their low levels of reactivity. In summary, three things were optimised for: 1) peak cytokine release (culture day 1), informative cytokines (IL-6, IL-10, IL-13, IL-17, IFN-γ, TNF-α and MIF) and the

antigens that gave the best reactivity (aCD3/aCD28, TT, PPD, LPS, PfSE). URBC was added as a negative control for PfSE.

7.6.2.2 Characteristics of donors

There were a total of 31 donors in 2008, 16 of who were in the weekly chloroquine group and 15 in the weekly placebo group. The baseline characteristics of these donors have already been described in section 7.2.3.

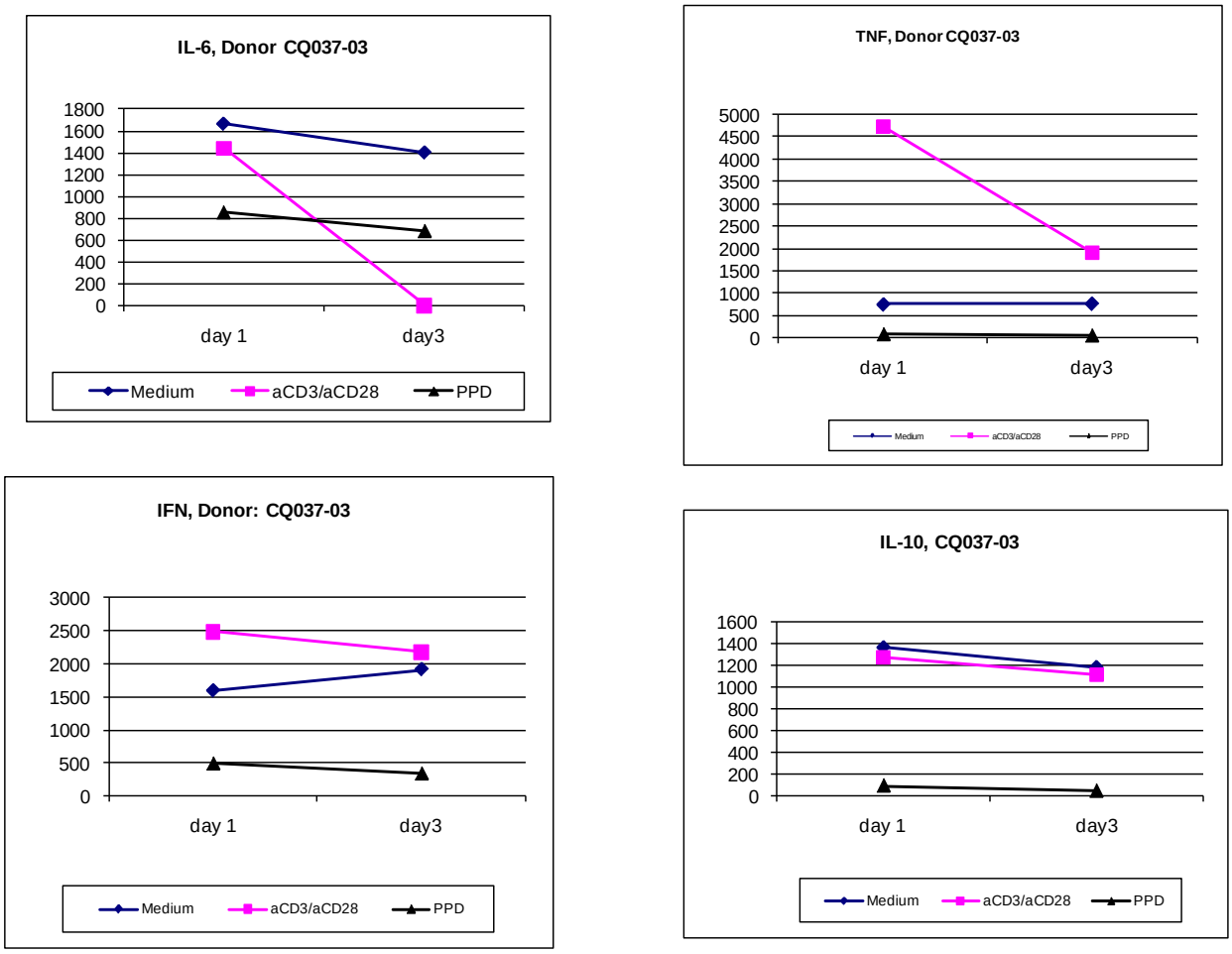


Figure 34: Comparison of cytokine response in Donor CQ037-03 between overnight whole blood culture and 3-day whole blood culture. The blue line represents the background cytokine concentration, pink line represents cytokine concentration in response to stimulation with aCD3/aCD28, while black line represents cytokine concentration in response to PPD stimulation. 'Day 1' represents supernatant harvested after overnight culture while 'day 3' represents supernatant harvested after 3 days of culture. In addition to the high background cytokine values, the overnight culture cytokine values were higher than the day 3 culture levels.

7.6.2.3 Baseline (day 3) cytokine responses

Since the randomisation into weekly chloroquine and weekly placebo did not commence until day 10, the day 3 responses in children randomised to both groups were combined and presented here. Table 20 shows the responses of the

different cytokines to stimulation by PfSE. All the cytokines were suppressed at day 3 except IFN-γ and IL-10.

Table 20: Net concentration of the different cytokines in response to stimulation of whole blood culture by PfSE at study day 3

Cytokines	IL-6	IL-10	IL-13	IL-17	TNF-α	IFN-γ	MIF
Plasma concentrations	-84.5 (-1449, 66.6)	333.6 (49.3, 635)	4.28 (-1.4, 23.2)	2.4 (-12.4, 7.4)	-6.1 (-26.1, 7.7)	524 (87, 1668)	10.32 (-1568, 689)

7.6.2.4 Chloroquine enhanced malaria-specific inflammatory responses and suppressed PPD responses

There was no difference in the baseline (study day 3) cytokine response between the chloroquine and placebo arms except IL-10 response where the response to LPS stimulation was significantly higher in the placebo arm than in the chloroquine arm (median IL-10 concentrations 300pg/ml (IQR: 100, 624) for the placebo group and -1374pg/ml (IQR: -1966, -569) for the chloroquine group; adjusted p-value = 0.03).

After overnight culture with antigens, the cytokine responses measured at day 45 showed an elevated TNF-α response to PfSE in the chloroquine group (median TNF-α concentration: 27.3pg/ml (IQR: -36.2, 153.7)) compared with the placebo group (median: -4.6pg/ml (IQR: -60.1, 6.55); p = 0.04) (Figure 35). The IFN-γ response to PPD stimulation on the other hand showed a significant decrease at day 45 in the chloroquine arm (median IFN-γ concentration: 279 pg/ml (IQR: 4.2, 1692)) compared with the placebo arm (median: 2459 pg/ml (IQR: 818, 8591); p = 0.02) (Figure 36). However, none of these differences in response remained significant after Bonferroni correction. All the other responses were not significantly different between the two groups.

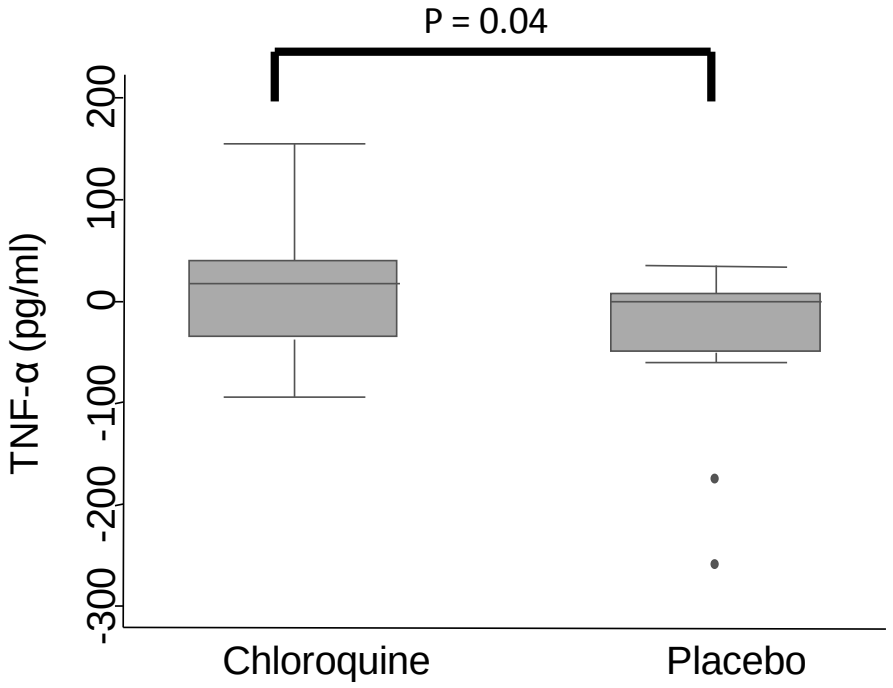


Figure 35: TNF-α response to PfSE stimulation after five weeks on weekly chloroquine (samples collected at study day 45)

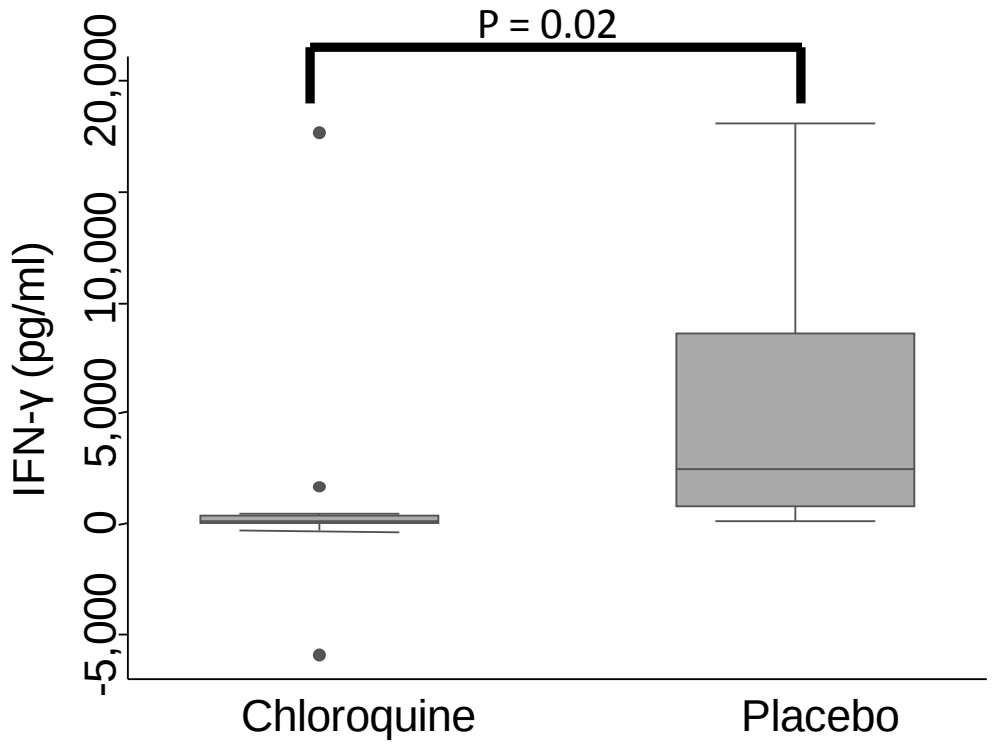


Figure 36: IFN-γ response to PPD stimulation after five weeks on weekly chloroquine (samples collected at study day 45)

7.6.2.5 Chloroquine enhanced innate recovery after five weeks of weekly chloroquine

The cytokine responses for days three and 45 were compared in the weekly chloroquine and weekly placebo randomisation groups. The results showed that for the chloroquine group, responses to the TLR4 agonist LPS at day 45 were higher for IL-10 (p=0.02), TNF-a (p=0.03) and MIF (p=0.02) when compared to the day 3 responses (Figures 37 – 39). The placebo group had comparable responses at both time points (days 3 and 45) for all cytokines and antigens (see Appendix 5). However, after correcting for multiple testing, these significant differences in the chloroquine group disappeared. Also, IL-10 response to PPD was significantly higher at day 45 in the chloroquine group (31pg/ml (IQR: -50, 712)) compared to the day three response (-858pg/ml (-1908, -495); p=0.006) (Figure 40). This difference remained significant after Bonferroni adjustment for multiple testing.

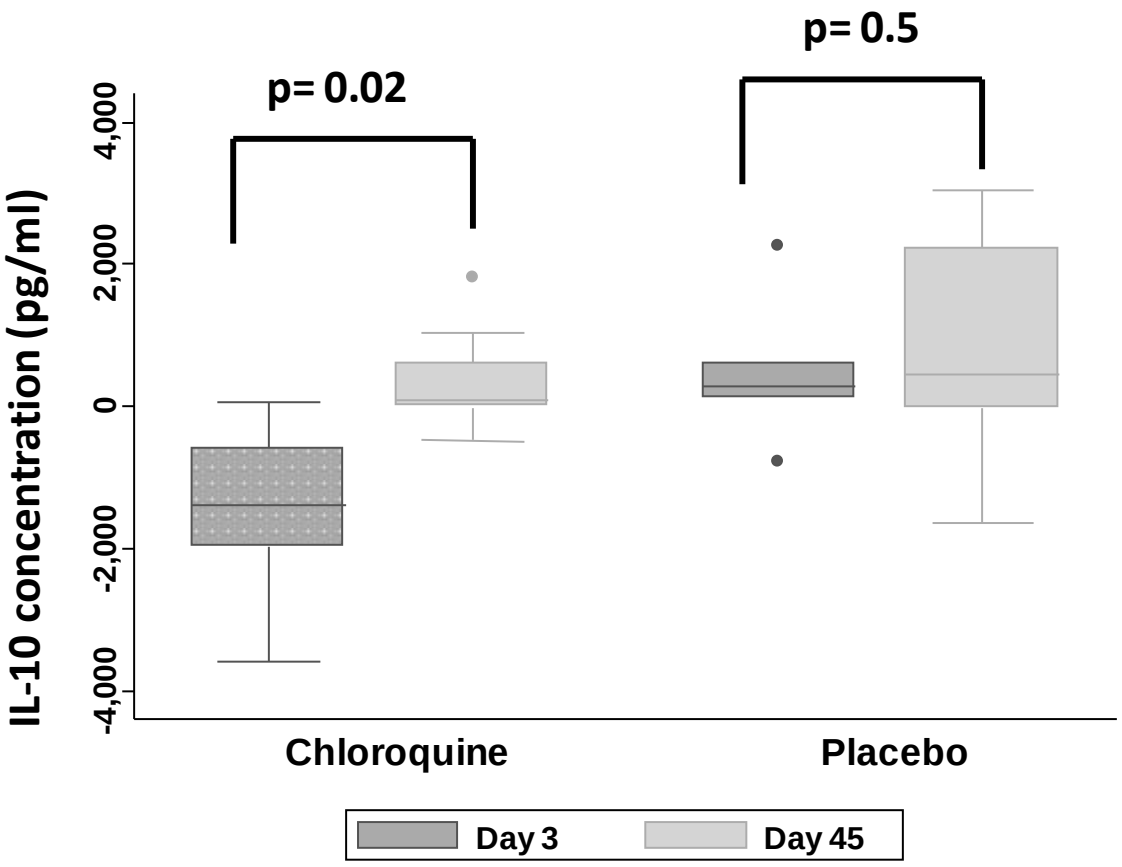


Figure 37: IL-10 concentration in response to LPS stimulation of overnight whole blood culture on study days 3 and 45 (Day 3 = sample collected on study day 3; Day 45 = samples collected on study day 45)

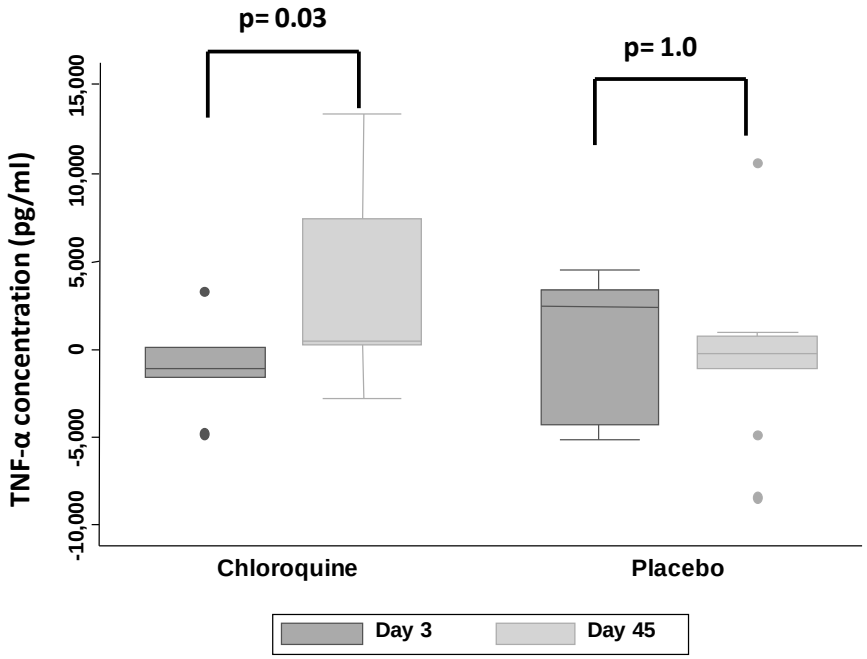


Figure 38: TNF- concentration in response to LPS stimulation of overnight whole blood culture (Day 3 = sample collected on study day 3; Day 45 = samples collected on study day 45)

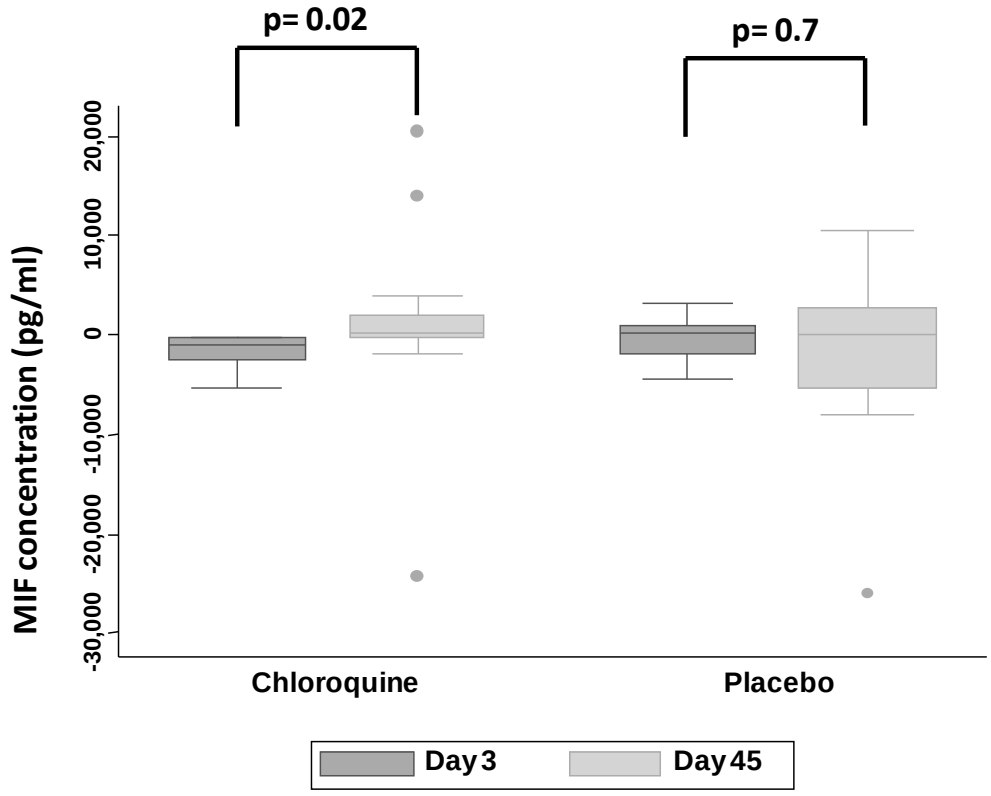


Figure 39: MIF concentration in response to LPS stimulation of overnight whole blood culture (Day 3 = sample collected on study day 3; Day 45 = samples collected on study day 45)

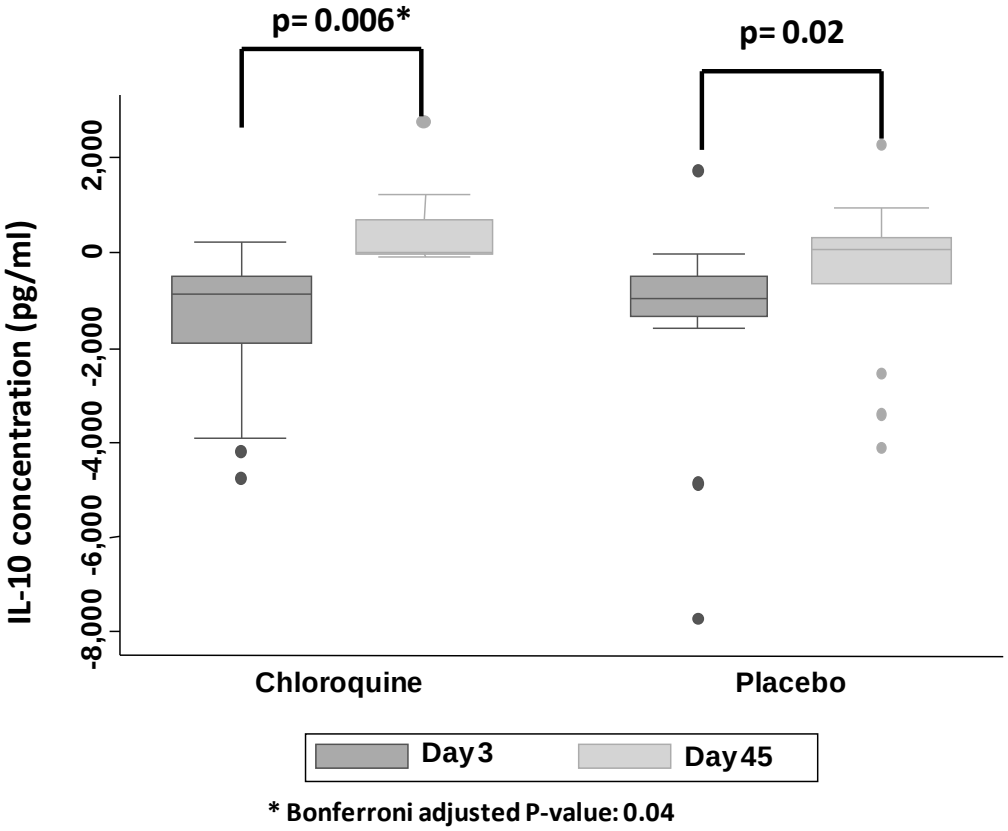


Figure 40: IL-10 concentration in response to PPD stimulation of overnight whole blood culture (Day 3 = sample collected on study day 3; Day 45 = samples collected on study day 45)

7.6.2.6 Chloroquine suppressed malaria specific responses

At day 45, the chloroquine group had lower IL-17 response to PfSE (-1.9pg/ml (IQR: -25.9, 10.6)) compared to the day three response (5.5 pg/ml (IQR: 13.1, 31.9); p=0.03). Interestingly, the PfSE stimulated Il-10 response was also lower at day 45 in the chloroquine group (7.7pg/ml vs 436pg/ml; p=0.007) but not in the placebo group. This difference remained significant after Bonferroni correction for multiple testing. There were no differences between days 3 and 45 responses in the placebo group for any of the conditions (see Appendix 8).

7.7 Other findings

7.7.1 Predictors of haemoglobin change

The predictors of Hb change among the study population were investigated. Simple regression analyses were first conducted with each of the predictor variables regressed on the final Hb individually (Table 21). Thereafter, all

variables achieving a p-value of 0.1 or below were included in a multiple regression analysis to investigate confounding.

Table 21: Univariate regression analysis to investigate the predictors of the final Hb

Parameter	Regression coefficient (95%CI)	P-value
Parasite count at day 0	3.5 (1.1, 5.8)	0.004
Sex	-0.4 (-3.3, 2.5)	0.8
Iron deficiency	-12.1 (-14.7, -9.5)	<0.001
HAZ	3.2 (1.8, 4.6)	<0.001
WAZ	2.6 (1.0, 4.2)	0.001
WHZ	0.1 (-1.6, 1.8)	0.9
BMI	-2.3 (-3.3, -1.3)	<0.001
Age in months	0.4 (0.3, 0.4)	<0.001
Treatment arm	1.2 (-2.1, 4.4)	0.5
Randomisation group	-0.5 (-3.4, 2.5)	0.8
Hb at day 0	0.6 (0.5, 0.6)	<0.001
Hb at day 3	0.7 (0.6, 0.8)	<0.001
Village	1.9 (0.9, 3.0)	<0.001
Presence of sub-microscopic parasitaemia	0.6 (-7.1, 8.2)	0.9
Year of study	-1.3 (-4.4, 1.8)	0.4

In building the multiple regression models, Hb at day 3 was dropped because of its strong correlation with Hb at day 0; and, while both were associated with the final Hb, the association between Hb at day 0 and the final Hb was stronger than the association between day 3 Hb and the final Hb. WAZ score was dropped because it was strongly correlated with both BMI ($r = 0.6$, $p < 0.001$) and HAZ ($r = 0.7$, $p < 0.001$). There was no association between BMI and HAZ ($r = -0.09$, $p = 0.08$). The final model consisted of all the variables that maintained significant associations with the dependent variable in a multiple regression model (Model 3). These were Hb at day 0 (i.e. at recruitment), presence of iron deficiency, height-for-age z score, and age (in months). Replacing 'presence of iron deficiency' with the individual measures of iron deficiency (MCV, MCH and ZnPP) at baseline in the model did not alter the model output. However, of the three parameters, only ZnPP at baseline maintained a significant association with the final Hb.

Model 3: Final multiple regression model showing the predictors of Hb in the study population

Source	SS	df	MS	Number of obs	=	352
				F(4, 344)	=	76.55
Model	31894.3138	4	7973.57845	Prob > F	=	0.0000
Residual	36144.1364	347	104.161776	R-squared	=	0.4688
				Adj R-squared	=	0.4626
Total	68038.4502	351	193.841739	Root MSE	=	10.206

Final Hb	Coefficient	Std. Err.	t	P>t	[95% Conf. Interval]	
					Lower	Upper
Day 0 Hb	0.438356	0.040961	10.7	<0.001	0.357793	0.518918
Presence of iron deficiency	-2.99798	1.307094	-2.29	0.022	-5.56881	-0.42716
Height-for-age Z score	1.483434	0.544954	2.72	0.007	0.411606	2.555262
Age in months	0.211431	0.034952	6.05	<0.001	0.142686	0.280176
_cons	53.1637	5.408511	9.83	<0.001	42.52611	63.80129

Figure 41 is a scatter diagram of the relationship between age and Hb. The final Hb was directly proportional to age of the children, measured in months. As can be seen from the figure, this pattern was similar in both groups (weekly chloroquine group is represented by the green dots, while the weekly placebo group is represented by the red crosses). The regression lines for both groups overlapped.

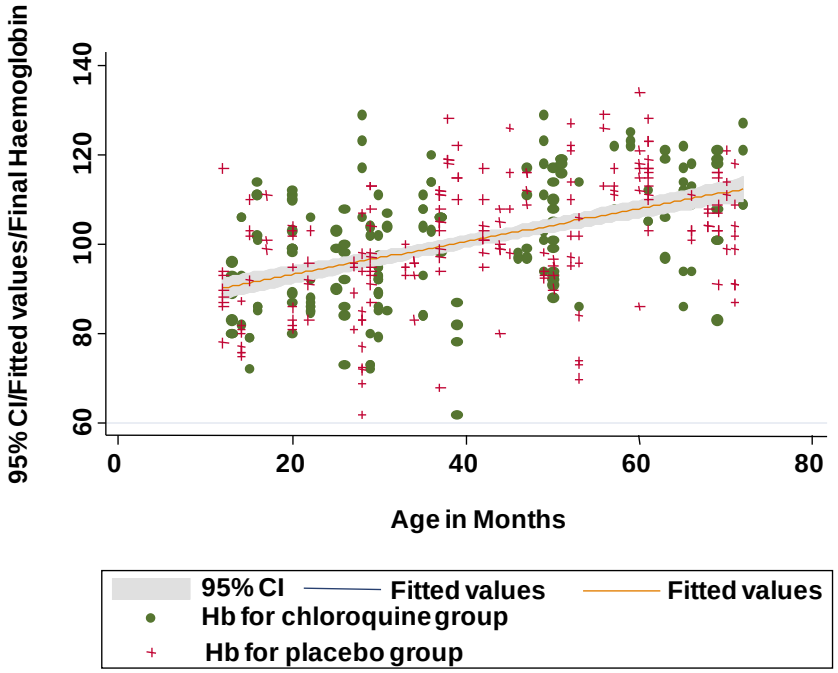


Figure 41: Correlation between age and final Hb

Figures 42 and 43 illustrate the relationship between day 0 Hb and day three Hb respectively, and the final Hb. Children with higher Hb concentrations at recruitment and at day three achieved a higher final Hb.

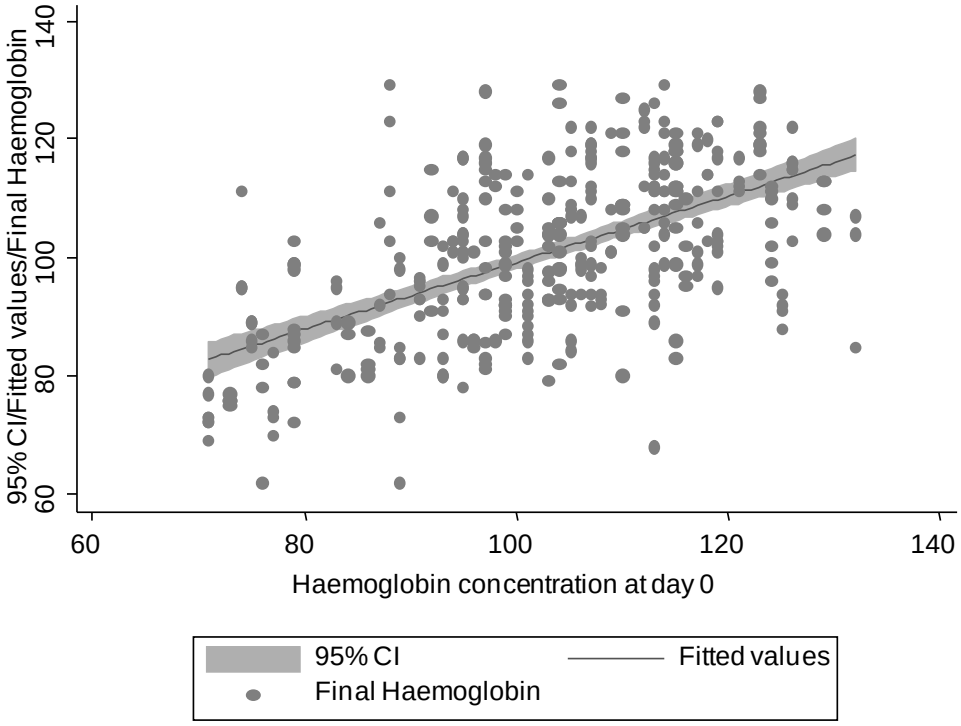


Figure 42: A regression fit of the association between Day 0 Hb and final Hb

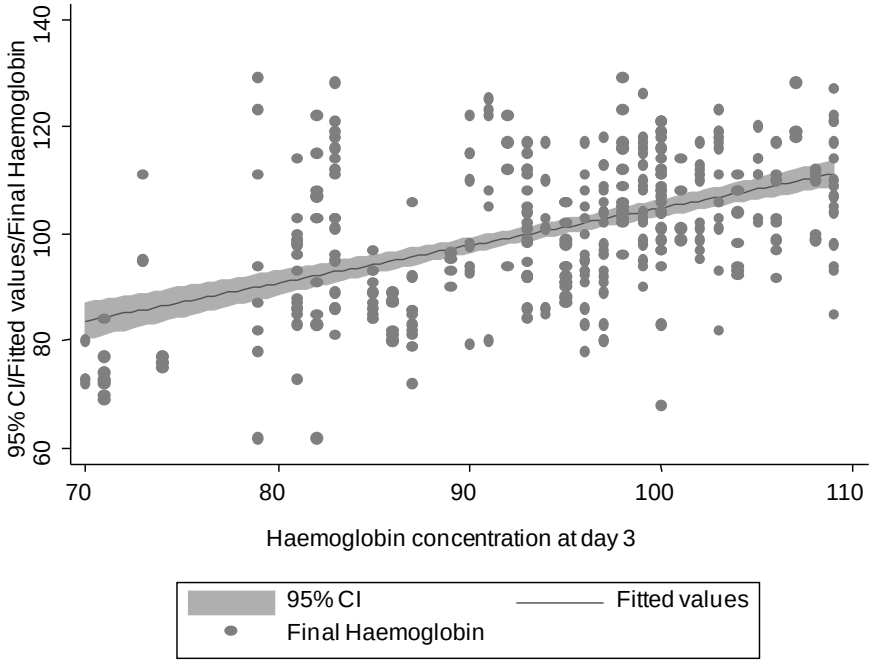


Figure 43: A regression fit of the association between Day 3 Hb and final Hb

The impact of iron deficiency on the final Hb among the population was also examined by comparing the final Hb in children who were iron deficient and the final Hb in children who were not iron deficient. The final Hb in children who were not iron deficient was 106.5 g/L (SD: 12.4) compared to 94.4 g/L (SD: 12.7) in children who were iron deficient; P <0.001.

7.7.2 Clinical correlates of iron deficiency

We explored the clinical parameters associated with iron deficiency in our participants by conducting a univariate analysis for each of the clinical parameters assessed. In the univariate analysis, children with iron deficiency were less likely to present with vomiting than children without iron deficiency (18.2% vs 35.4%, p = 0.06); but were more likely to present with difficulty with breathing (34.1% vs 14.6%, p=0.03) and were more likely to be diagnosed with coryza (23.8% vs 8.7%, p=0.05) than children without iron deficiency. When these three parameters were included in a multiple logistic regression model with iron deficiency as the dependent variable, only the presence of difficulty with breathing at presentation remained significant (odds ratio (95%CI): 3.1 (1.1, 9.0), p=0.03). Although children with iron deficiency were three times more likely to be diagnosed with coryza than children without iron deficiency, this only achieved marginal significance after adjustment (OR (95%CI): 3.1 (0.9, 11.4), p=0.08).

7.7.3 Sub-microscopic parasitaemia

The incidence of sub-microscopic parasitaemia across the study period was 15.1%. The same proportion of children (15%) from the CQ-SP treatment group and the ACT treatment group had sub-microscopic parasitaemia (P=0.99). There was no significant association between randomisation into weekly chloroquine or weekly placebo and the presence of sub-microscopic parasitaemia. Only the parasite count at diagnosis was associated with the presence of sub-microscopic parasitaemia. One log increase in parasite count increases the odds of having sub-microscopic parasitaemia by 1.6 (P=0.005). Sub-microscopic parasitaemia was commonest in the first seven days after diagnosis and by day 15, most of these had cleared. For the majority of children who still had sub-microscopic parasitaemia after day 15, these were of very low level parasitaemia (one to two parasites per microliter). A few cases of high level parasitaemia were detected at day 30 (three children), day 45 (one child), day 70 (two children) and day 90 (one child). Figure 44 describes the trend in sub-microscopic parasitaemia

across the study period. As the prevalence in sub-microscopic parasitaemia fell, the change in Hb increased. This trend was similar for both arms. However, in a multiple linear regression using the random effects model, no association was found between the presence of sub-microscopic parasitaemia and the final Hb.

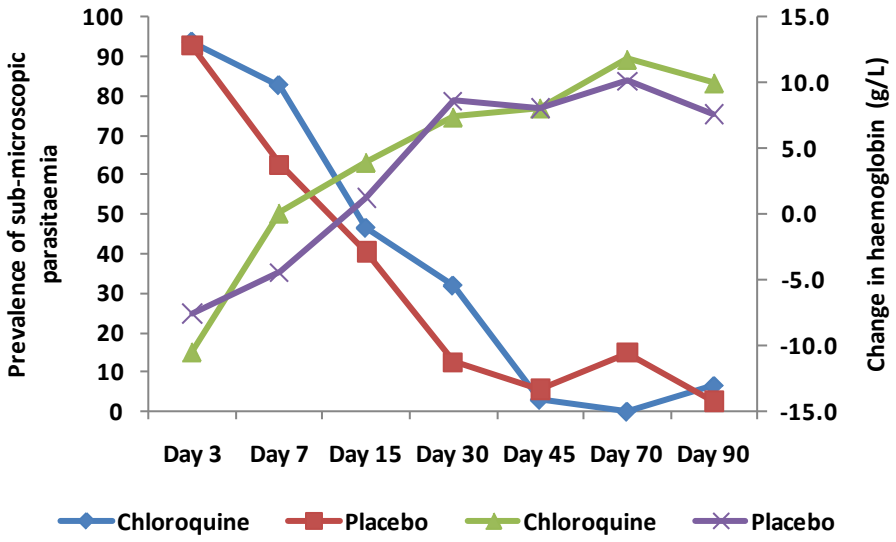


Figure 44: Prevalence of sub-microscopic parasitaemia across the study period and its relationship to Hb change

As already noted, the parasitaemia were mostly between 1 and 2 parasites per microlitre. Four children had qPCR parasitaemia levels above 500/µL between study days 30 and 90. Of these four, three were initially treated with chloroquine and one child initially treated with ACT. Two of the four children were in the weekly placebo group and the other two were in the weekly chloroquine group. The parasite counts at diagnosis for the four children are shown in table 22.

Table 22: Parasite count (by qPCR) in the four children with parasitaemia above 500/µL between days 30 and 90

				Parasite Count (per µL)							
Subject ID	Treatment‡	AGE (mo)	Sex	Day 0	Day 3	Day 7	Day 15	Day 30	Day 45	Day 70	Day 90
A12	CQ-SP	29	M	39014	3	2	0	2185	0	0	0
A26	CQ-SP	59	F	27089	1	0	0	0	0	3595	0
A67	CQ-SP	44	F	249333	2191	--	735	6661	--	--	21336
A100	ACT	20	M	45589	7	--	11	6533	15158	3071	--

‡CQ-SP represents children who were initially treated with chloroquine-sulphadoxine-pyrimethamine before randomisation to weekly chloroquine and weekly placebo, while ACT represents children who were initially treated with co-artemether.

The mean Hb change of the three children with qPCR parasite count above 500/µL (significant parasitaemia) was significantly lower than the mean Hb change in children with one to two parasites/µL or no parasites at day 30 (-5.0g/L vs 8.5g/L; mean Hb difference (95%CI): 13.5g/L (-0.3, 27.3); p = 0.06). Similarly, the absolute Hb concentration at day 30 of the three children with significant parasitaemia was significantly lower than in the children with few or no parasites (82.3g/L vs 102.8g/L; p = 0.01).

We explored the association between the urinary neopterin and qPCR parasite count in the children with parasitaemia at day 30. At day 30, 12 children had one parasite per microlitre, five children had two parasites per microlitre and three children had parasite counts above 500/µL (Table 22). Because both neopterin and parasite counts were skewed, both were transformed by taking their natural logs before regression analysis. Regressing neopterin concentration at day 30 on qPCR parasite count at day 30 (used as a continuous variable) showed a strong association between parasite count and urinary neopterin (regression coefficient: 0.2 (95%CI: 0.08, 0.33), p = 0.003; adjusted R^2 = 0.4091). Figure 45 is a graphical display of the correlation between the log of neopterin at day 30 and the log of the qPCR parasitaemia at day 30.

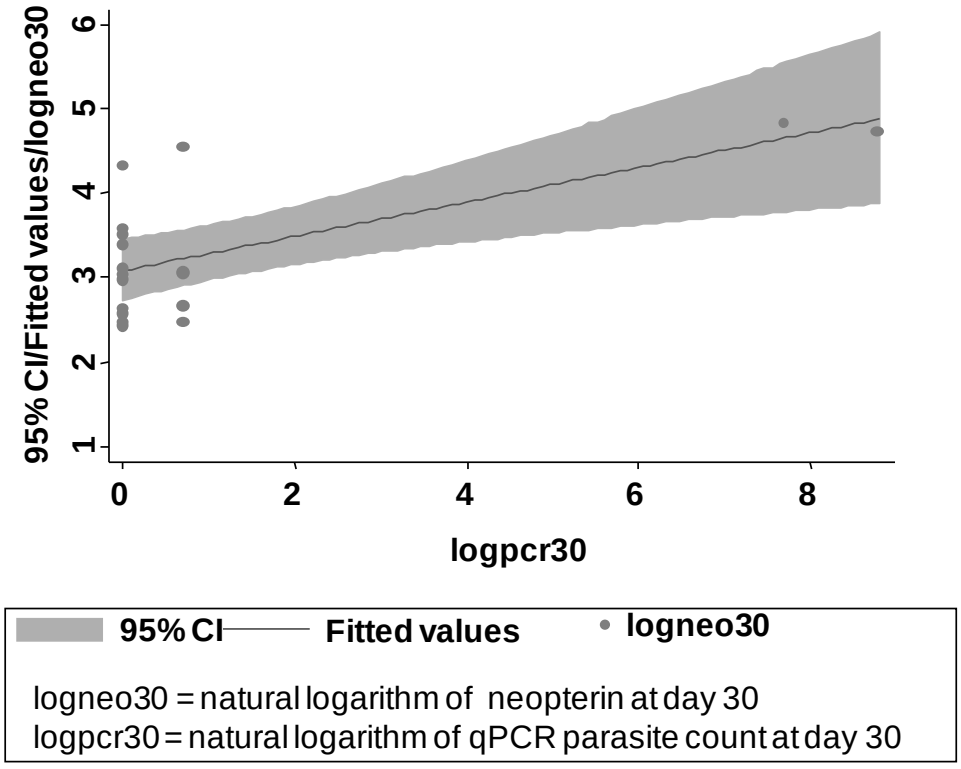


Figure 45: A regression fit of the association between log neopterin at day 30 and log of qPCR parasite count at day 30; pairwise correlation coefficient (r) = 0.7, p = 0.003

The groups were further sub-divided into three – children with no parasitaemia at day 30 (coded '0'), children with one or two parasitaemia per microlitre (coded '1') and children with parasitaemia above 500/μL (coded '2'). The parasite count was then entered into the regression model as a discrete variable. The output showed that at day 30, the urinary neopterin in children with one to two parasites/μL was not significantly different from the levels in children without parasitaemia. However, the neopterin concentrations in children with parasite counts above 500/μL were significantly higher than those without parasitaemia (Model 4).

Model 4: regression output of the association between urinary neopterin concentration and parasite count with the parasite count categorised into 'no parasite', 1-2 parasite/μL, and parasite count above 500/μL

Source	SS	df	MS	Number of obs	=	78
				F(4, 344)	=	7.16
Model	15049.98	2	7524.991	Prob > F	=	0.0014
Residual	78786.93	75	1050.492	R-squared	=	0.1604
				Adj R-squared	=	0.138
Total	93836.91	77	1218.661	Root MSE	=	32.411

Day 30 Neopterin‡	**Coefficient**	**Std. Err.**	**t**	**P>t**	**[95% Conf. Interval]**	
					Lower	Upper
_Ipcr30_1	-5.91227	9.340986	-0.63	0.529	-24.5205	12.69592
_Ipcr30_2	85.47639	23.29093	3.67	<0.001	39.07848	131.8743
_cons	34.52361	4.149842	8.32	<0.001	26.2567	42.79052

‡pcr30_1 represents the group with parasite of 1-2/μL at day 30 by qPCR; pcr30_2 represents the group with parasite of >500/μL at day 30 by qPCR; the comparison group was the group with no parasites at day 30.

Introducing actual Hb at day 30 into the model did not alter the association; but it demonstrated that there was no association between Hb at day 30 and the urinary neopterin (p=0.9). Similarly, introducing change in Hb at day 30 did not alter the association between urinary neopterin and parasite count. Also, controlling for randomisation group in the model did not alter the association. There was no association between qPCR parasite count at day three and urinary neopterin concentration at day three. Only one child had a qPCR parasite above 500/μL at day three.

7.8 Summary

In summary, in the setting of this study, giving children with mild malaria anaemia chloroquine at a dose of 5mg/kg/week following microscopically confirmed parasitological cure did not confer any advantage to bone marrow recovery compared to children who received placebo. Changes in markers of iron status, bone marrow response and macrophageal activation did not differ between the intervention group and the placebo group. The independent predictors of Hb concentration were the age of the child measured in months, the Hb at day 0, height-for-age z score and the presence or absence of iron deficiency.

8 Chapter 8 – Discussion

8.1 Summary of key findings

8.1.1 Weekly chloroquine administration to children with malaria anaemia over three months did not improve haemoglobin recovery compared with children receiving placebo.

Chloroquine is a known anti-inflammatory agent which has been used for decades to treat inflammatory conditions like rheumatoid arthritis and systemic lupus erythematosus. Malaria is essentially a systemic inflammatory condition. The release of inflammatory mediators plays a major role in the pathogenesis of malaria-induced anaemia. It is therefore plausible to hypothesise that chloroquine, by suppressing plasmodium-induced inflammation, could enhance erythropoietic recovery. To test this hypothesis, I compared two groups of children – one group received weekly chloroquine and the other group weekly placebo after complete eradication of the parasitaemia as determined by microscopy. The primary outcome measure was the change in Hb at days 30 and 90 from the day three Hb levels. There was no significant difference in the Hb change between both arms. The data was interrogated further by the use of random effects model, a robust regression model that takes into account random measurement errors and missing data. This regression analysis confirmed that giving the children weekly chloroquine at a dose of 5mg/kg body weight did not improve Hb recovery after acute malaria.

8.1.2 There were no differences in the changes in MCV, MCH and ZnPP over 90 days between children who received weekly chloroquine and those who received weekly placebo

It is possible for the intervention to affect the body's iron status without obvious changes in the Hb. To explore this possibility, I compared the changes in MCV, MCH and ZnPP – three markers of iron status – between the two intervention arms. This analysis also revealed no difference in the weekly chloroquine group compared with the weekly placebo group. Although the median ZnPP values at day 30 was lower in the chloroquine arm compared with the placebo arm, this

difference only achieved marginal significance and may have been as a result of chance.

8.1.3 Reticulocyte response in the weekly chloroquine arm was similar to that in the weekly placebo arm both in pattern and magnitude.

Reticulocyte count helps to monitor bone marrow response. A better bone marrow response in either of the arms will be indicated by a higher reticulocyte percent. The findings from this study indicate that there was no difference in the reticulocyte response between the weekly chloroquine and weekly placebo arms. While there was a higher increase in the day 30 reticulocyte percent in the chloroquine arm than in the placebo arm, this did not reach statistical significance. More-over, the pattern of response between the two groups were similar.

8.1.4 Changes in markers of inflammation did not differ significantly between the chloroquine and placebo groups

Neopterin was used as a proxy measure of macrophageal activation. Studies have shown that the concentration of neopterin rises in response to macrophageal activation; and declines with resolution of the inflammation. If chloroquine suppressed macrophageal activation as hypothesized in this study, one would expect greater decrease of neopterin in the chloroquine arm compared to the placebo arm. The effects of the intervention on malaria-induced inflammation were compared by measuring the urinary neopterin at days 3, 15 and 30; and by measuring cytokine production in the supernatants of stimulated whole blood samples. Both measures indicated that there was no difference in responses between the chloroquine and placebo arms. During the acute malaria phase, and prior to the commencement of weekly chloroquine and placebo administration, the urinary neopterin concentrations were markedly elevated. The levels fell dramatically in the two groups at day 15 and remained low up to day 30. At each of the measurement points, there was no significant difference between the levels in children on weekly chloroquine and those on weekly placebo. The sharp fall in neopterin concentrations between day three and day 15 would suggest that by day 15, the inflammatory process had almost completely

resolved, in which case the inflammatory insults to the erythropoietic system would be expected to be less by this time. As noted in chapter three, malaria is essentially an inflammatory illness and plasmodium-induced inflammation plays an important role in the pathogenesis of malaria anaemia. Therefore, if the malaria-induced inflammation had subsided by day 15, the impact of the weekly chloroquine administered would be expected to be less; which could explain the lack of any difference noted in the Hb change in the weekly chloroquine and weekly placebo arms. Unfortunately, cytokine values for days 15 and 30 were not available for comparison. However, for most of the cytokines assessed, there were no significant differences between the chloroquine and placebo arms at day 45.

Acute malaria was associated with suppression of IL-6, IL-13, IL-17, TNF-α and MIF, and elevation of IFN-γ and Il-10 in response to whole blood stimulation with the malaria specific antigen, PfSE. At day 45, chloroquine administration was associated with elevation of TNF-α response to PfSE and a suppression of IFN-γ production in response to PPD. The small sample sizes (16 for chloroquine and 15 for placebo) might have contributed to the inability of these differences to remain significant after correction for multiple testing. Administration of weekly chloroquine also significantly enhanced IL-10 response to PPD stimulation at day 45 compared to day three, and suppressed IL-10 response to PfSE stimulation. These differences remained significant even after correction for multiple testing. These results clearly suggest that IL-10 concentration is elevated in acute malaria.

8.1.5 Children treated with chloroquine-sulphadoxine-pyrimethamine have better haemoglobin recovery in the long term than children treated with co-artemether

The possible effect of treating a child with CQ-SP on macrophageal iron loading and release was explored by comparing the Hb responses in the placebo arms of the 2007 study. Children in these arms did not receive any weekly chloroquine; therefore, any differences in Hb response seen between the CQ-SP and ACT treated children will have arisen from the effects of the treatment given. From figure 32, it can be seen that during the first week after malaria diagnosis, the initial fall in Hb was greater in the CQ-SP arm but the Hb recovery thereafter

was also greater in the CQ-SP arm compared with the ACT arm. However, as indicated by the error bars, there was considerable overlap in the Hb responses in the two comparison groups. That the differences noted in Hb response were not significant was confirmed in a random effects model which showed that Hb response in children treated with CQ-SP was not significantly different than in children treated with ACT. The initial greater fall in Hb in the CQ-SP arm compared with the ACT arm might indicate that ACT clears parasitaemia faster than CQ-SP; but the better erythropoietic response of the CQ-SP treated group, even though not statistically significant would suggest a more favourable effect of CQ on macrophageal iron flux. This is important considering that in the acute malaria phase, the dose of chloroquine given (10mg/kg/day over 3 days) was much higher than the recommended daily anti-inflammatory dose (3-4mg/kg/day).

8.1.6 Predictors of haemoglobin change

The predictors of Hb change in this study were Hb at day 0 (i.e. at the time of diagnosis), presence of iron deficiency, height-for-age z score, and age of the child (in months). Together these parameters explained 46% of the variation in Hb at the end of the study. Children with higher Hbs at diagnosis had better Hb responses than those with lower Hbs. Older children also had better Hb responses. A one month increase in age of the child resulted in an increase in Hb of 0.21g/L. The presence of iron deficiency led to a 12.1g/L decrease in Hb by the end of the study. The clinical correlates of iron deficiency identified in this study were the presence of difficulty with breathing, a diagnosis of coryza and the absence of vomiting in a child with confirmed malaria. There was a three-fold higher prevalence of coryza in children with iron deficiency than in children that were not.

8.1.7 Incidence of sub-microscopic parasitaemia was low among the study population

Contrary to expectations, the incidence of sub-microscopic parasitaemia was very low in this study. When present, the levels were generally low – between one and three parasites per microliter. There was no difference in the incidence of sub-microscopic parasitaemia in children treated with CQ-SP compared with

those treated with ACT; there was also no difference in the incidence of sub-microscopic parasitaemia in children given weekly chloroquine compared with those given weekly placebo. The low incidence of sub-microscopic parasitaemia was consistent with the lack of persistence of inflammation among our study participants as suggested by the urinary neopterin levels. Model 4 clearly showed that children with significant sub-microscopic parasitaemia had higher levels of neopterin compared with children with no sub-microscopic parasitaemia and children with one to two parasites/μL. The prevalence of sub-microscopic parasitaemia appeared to be inversely related to the change in Hb (Figure 44), although in a multiple regression analysis, presence of sub-microscopic parasitaemia was not a determinant of the final Hb. It is likely that with a larger sample size, this relationship could become significant. This is suggested by the finding that mean Hb change and absolute Hb in the children with significant sub-microscopic parasitaemia were significantly lower at day 30 than the levels in the children with no sub-microscopic parasitaemia or just one to two parasites/μL.

The Ethics Committee had raised concerns about the risk of drug resistance in the context of chloroquine monotherapy and the risk of children developing significant parasitaemia after randomisation. The findings from this study however showed that this risk was minimal. There were only four second malaria episodes – two in the weekly chloroquine group and two in the weekly placebo group. Also the risk of sub-microscopic parasitaemia did not depend on the initial antimalarial treatment or the randomisation group. The only determinant of having sub-microscopic parasitaemia was the parasite count at diagnosis, with children having higher parasite load at diagnosis being at greater risk of having sub-microscopic parasitaemia. The progression of sub-microscopic parasitaemia in the four subjects that remained parasitaemic by qPCR after day 30 suggests however that there is still a small risk of drug resistance and the development of significant parasitaemia in these children. Apart from subject A26 (Table 22), the other three subjects continued to have low grade sub-microscopic parasitaemia from day three until day 30 when there was a ‘flare up’.

8.2 Implications of the findings

I had hypothesized that through its anti-inflammatory and immunomodulatory actions, chloroquine would reduce macrophageal iron sequestration and enhance

early remobilisation of body iron stores, thus increasing the concentration of iron available for erythropoiesis. I also anticipated that this anti-inflammatory action will reduce malaria-induced bone marrow suppression, enhancing the ability of the bone marrow to respond to erythropoietic stimuli. I had hoped that all these effects would translate into an increase in Hb in the weekly chloroquine arm compared with the group given weekly placebo.

The lack of a significant difference in the Hb change between the weekly chloroquine and weekly placebo arms would suggest that my intervention did not enhance erythropoiesis as hypothesised. This apparent lack of effect could have resulted from the use of prophylactic chloroquine dose (5mg/kg/week) rather than the currently recommended anti-inflammatory dose of 3-4mg/kg/day. At diagnosis, the children were given the approved therapeutic dose of 30mg/kg over three days, which in the short-term could reduce macrophageal iron loading, in addition to the antimalarial effects. Thereafter, the smaller dose was administered for three months. Considering the potential adverse effects of high dose chloroquine, particularly retina damage, it was desirable to use the lower dose which would also help me to assess the safety of long-term chloroquine administration in children with malaria anaemia. As far as I know, this is the first study to have attempted to use chloroquine in the management of children with malaria anaemia. Therefore without appropriate safety data, it would have been unethical to use a higher weekly chloroquine dose for these children.

The small sample size might also have affected my ability to find an effect. The calculated sample size that would have enabled me detect a difference of 7.5g/L was 260 if I had continued with the 2x2 study design and 130 for the two arm design. However, I had only 96 participants overall or 37% of the expected sample size. If the 27 subjects that were initially treated with CQ-SP were removed, only 69 subjects would remain for the two-arm study or 53% of the expected sample size. Thus, the power of the study to detect any differences was greatly reduced. The small sample size also limited my ability to adequately assess the changes in other outcomes such as cytokines. The cytokines were assessed in only 31 participants out of an estimated sample size of 130.

Another possible reason for the lack of effect of chloroquine supplementation seen in this study is the high prevalence of iron deficiency among the

participants. Children with pre-existing iron deficiency would be expected to respond less well to chloroquine supplementation as it will limit the amount of iron available for re-mobilisation. This is important because iron deficiency was shown in this study to be one of the independent predictors of Hb change at both study days 30 and 90. Thus to adequately test my hypothesis, it would have been desirable to exclude children with pre-existing iron deficiency from the analysis. However, excluding these children from the analysis would have further reduced the power of the study.

In summary, the implications of these observations on the three effects I originally set out to achieve are as follows:

1. Inhibition of macrophageal iron loading through the initial use of chloroquine at the therapeutic dose of 30mg/kg over three days: I could not prove or disprove an effect because of the small numbers of participants.

2. Inhibition of further episodes of malaria by the long-term use of chloroquine at a dose of 5mg/kg/week: I did not find any difference in the prevalence of sub-microscopic parasitaemia between the chloroquine and placebo arms; but there were too few numbers of recrudescence or second malaria episodes to assess this difference.

3. Anti-inflammatory effects through the use of chloroquine at a dose of 5mg/kg/week: I did not detect any effect of chloroquine on Hb; and the effects on markers of inflammation (neopterin and cytokines) were too variable primarily because of small numbers. It is also likely that my ability to adequately assess this outcome might have been affected by the small chloroquine dose used; the dose being too low for anti-inflammatory action. There is therefore insufficient data to determine the effects of weekly chloroquine on the markers of inflammation. However, as already discussed, there is no evidence on the safety implications of long-term higher doses of chloroquine for children with malaria anaemia.

Other implications of the results:

1) Chloroquine given at a dose of 5mg/kg/week does not seem to enhance Hb recovery post malaria.

2) Giving children weekly chloroquine at a dose of 5mg/kg/week for 90 days after a malaria episode is safe.

3) Hb recovery in children with malaria anaemia is better in older children than in younger children. Therefore, while anaemia in all children with malaria should be viewed with seriousness, children aged 3 years and below require special attention.

4) Pre-morbid Hb is a strong predictor of the ability of a child to recover from malaria-associated anaemia, with higher pre-morbid Hb being associated with better recovery.

5) Co-existing iron deficiency reduces the ability of a child to recover from malaria anaemia.

6) The long term effect of treating children with CQ-SP on Hb recovery was not different from the long term effect of treating the children with ACT.

8.3 Findings in the context of literature

There is abundant evidence in the literature of the use of chloroquine in the treatment of several diseases outside its antimalarial use[348-349] . Wallace[349] noted 30 non-infectious disorders for which chloroquine was used as therapy. These conditions included blood and connective tissue disorders, and cutaneous conditions. Earlier studies in The Gambia[308], Nigeria[309], Mali[310] and Liberia[311] have all shown that chloroquine chemoprophylaxis in children significantly increased Hb compared with use of other antimalarials or placebo (see Chapter 4). Similarly, use of chloroquine chemoprophylaxis in pregnant women in Cameroon and Burkina Faso[313], Uganda[314] and Thailand[350] were all associated with improved Hb status when compared with controls. The failure of this study to demonstrate an effect could have been because of the high prevalence of iron deficiency in my cohort. Even though many of these children were iron deficient, they did not receive iron therapy, while the pregnant women received

iron therapy in addition to chloroquine chemoprophylaxis. Therefore it is difficult to determine that the chloroquine prophylaxis was responsible for improvement in Hb.

The use of prophylactic dose (5mg/kg/week) to investigate the anti-inflammatory effects of chloroquine might have also contributed to our inability to detect an effect. In one report, Drenou et al[351] observed that one adult patient with sideroblastic anaemia (a condition characterised by reduced incorporation of iron into haem causing anaemia in the presence of sufficient or high iron load) responded very well to a daily dose of 300mg chloroquine for two months but relapsed when the chloroquine was stopped. The patient's sideroblastic anaemia was eventually successfully controlled for six years on 100mg daily dose of chloroquine without need for blood transfusion. Although the mechanism for this effect was not explored, this finding suggested that chloroquine is able to act at some point along the haem synthesis pathway to enhance erythropoiesis. In this patient, iron was available but was not being incorporated into haem, a situation that was reversed by chloroquine. I had hypothesized that by blocking iron delocalisation, chloroquine could enhance iron utilization in malaria anaemia and thereby enhance erythropoietic recovery. It is important to emphasize that the dose of chloroquine used for immunomodulatory and anti-inflammatory effects are usually much higher than the dose used in this study.

Chloroquine is a lysosomotrophic agent and therefore inhibits iron uptake by cells. In real life, this iron-loading restriction will not be limited to macrophages, but could also involve iron-transport proteins like transferrin. This implies that chloroquine could actually have a negative effect on Hb recovery in children given weekly chloroquine. However, the finding that there was no difference between children given weekly chloroquine and those given weekly placebo means that while chloroquine did not confer any benefit, it did not also pose any risk to the study participants.

The Hb in the study participants fell between days 0 and day seven and gradually recovered, reaching the pre-morbid Hb levels by day 30. This level was maintained until day 90. This pattern was the same for both groups. Studies that looked at the therapeutic efficacy of chloroquine have also found an initial fall

in Hb in the first one week after diagnosis followed by a gradual recovery. However, not all the studies reported a recovery to the Hb level at recruitment. Hamer et al[352] found a return to the pre-treatment Hb in their cohort while Ekvall et al[353] reported a lack of improvement in Hb in the children treated with chloroquine. These differences are understandable in light of the various determinants of Hb change following malaria. For example, I found that age and presence of iron deficiency were strong determinants of Hb recovery after malaria. Therefore differences in these variables among the different studies could result in differences in outcome. In the Ekvall study[353], their participants were aged five to 36 months while I studied children aged 12 to 72 months. It is interesting that my results were similar to those of Hamer et al[352]who also studied older children.

I had hypothesized that chloroquine will impede macrophageal iron sequestration during the acute clinical episode and improve iron availability for the process of erythropoiesis. As was discussed in Chapter six, macrophageal activation is associated with neopterin release, increasing the circulating neopterin levels and neopterin excretion in the urine; hence the use of urinary neopterin as a marker of immune activation. It has also been shown that urinary excretion of neopterin is markedly increased in individuals infected with malaria, and corresponds with concurrent activation of T cells and macrophages[345, 354-355]. To test the effect of administering weekly chloroquine on macrophageal activation, I measured the urinary neopterin at days 3, 15 and 30. I expected to see a more rapid decline in neopterin level in the weekly chloroquine group than in the weekly placebo group; but no such difference was found between the two comparison groups. This finding however, was not surprising. The reduced neopterin excretion in the two groups was so dramatic that by day 15, the levels were near zero in both groups. With such low levels, it would be difficult to measure any difference at that stage. Figure 33 suggests the possibility that because of the large interval between the first two neopterin measurements, the progression of the changes in macrophage activation may have been missed. Indeed, Brown et al[355] showed that in a group of volunteers experimentally infected with *plasmodium* parasites, the timing of maximum neopterin excretion coincided with the treatment and elimination of parasitaemia. In this study, this would coincide with study days 0 to three. Thus by day 15, the macrophageal activation would have completely resolved. These

results suggest therefore, that it would be more informative to measure neopterin more frequently in the first week after diagnosis of malaria. The results further suggest that measuring urinary neopterin after day 15 was of little value to the study, probably due to the very small numbers of sub-microscopic parasitaemia among our cohort. It is important however to note that the high concentration of neopterin excretion noted in this study at day three (during the acute phase of the malaria) is consistent with several studies which found raised neopterin in association with acute malaria[345, 354-355].

The participants were followed up for 90 days because several reports had suggested that Hb continues to fall even after successful malaria treatment primarily due to continuing inflammation[85] . In this study however, I have not found any evidence of persistent inflammation, judging by the fact that urinary neopterin, which was used as a marker for macrophageal activation returned to normal values by day 15 of the study and remained at this low level up till day 30. In a group of 26 Zambian children treated for cerebral malaria and followed for 30 days, Biemba et al[356] reported that in six children with persistent anaemia at day 30, the neopterin concentration remained elevated compared with the levels in those whose anaemia had resolved. However, neopterin was measured only up to day seven in those subjects. In line with this report, I found elevated neopterin levels in three subjects who remained parasitaemic by day 30. In addition, I also found that both the absolute Hb and the Hb change at day 30 were lower in these children compared with children who had either no parasitaemia or had parasite counts of one to two per microlitre.

The similarities in the cytokine responses at days 3 and 45, particularly the pro-inflammatory cytokines, would suggest persistence of the inflammatory process. But caution is required in interpreting the findings as the absolute cytokine levels were very low, with most of the responses being lower than the background values (suggesting suppression of cytokine production). Thus at this low cytokine concentration, it might be difficult to detect any difference between the two comparison days.

Studies have shown a wide variability in cytokine responses to stimulation in the presence of malaria infection. The response of cytokines to chloroquine treatment during malaria was more variable. While IL-10 (an anti-inflammatory

cytokine) increased in response to LPS, PPD and TT stimulation, it decreased in response to PfSE stimulation in both the chloroquine and placebo arms. For the pro-inflammatory cytokines, while IL-17 production decreased at day 45, TNF-α and IFN-γ increased in the chloroquine arms. No change was noted in the placebo arms.

Jason et al[131] suggested that the immune response to malaria was more pro-inflammatory in children. They further reported that IL-10 response was specific to malaria. Table 20 indicate that of the seven cytokines assessed, only IL-10 and IFN-γ were elevated in response to malaria-specific antigen. The responses of the other antigens were more variable. Balal et al[357] reported a decline in anti-inflammatory cytokines in adult malaria patients treated with 25mg/kg of chloroquine over three days. However, no significant difference between day three and day 45 cytokine levels was found for most of the cytokines considered. Jason et al[131] had provided data suggesting that cytokine responses in adults varied from those of children; so the differences between the findings from this study and those of Balal et al[357] might relate to the age difference between our study populations.

8.4 Study limitations

Conscious efforts were made to ensure that this study was as rigorous as possible, taking care to ensure that all the necessary logistics are in place to test the hypothesis. Several unforeseen circumstances might have affected the outcome of the study. Of particular importance are:

1) Low malaria incidence during the two years the study was conducted;

2) The low dose of chloroquine used in the weekly supplementation phase of the study; and

3) The change in malaria treatment policy by the Gambian government.

Some of these situations can be corrected in the design of future studies; but there are some situations that are entirely beyond the control of the researchers. One classic example of such unavoidable circumstance was the declining incidence of malaria in The Gambia. I had calculated that based on previous population-based studies conducted in the district, I would have enough

participants to complete my sample size even after removing children who might not meet the inclusion criteria. This dramatic fall was therefore unanticipated. Based on previous studies conducted at the centre used for this study, the expected malaria incidence was about 34%. Although there were suggestions of declining malaria incidence in the Gambia (personal communications), I did not anticipate the dramatic decline witnessed in this study. A malaria incidence of at least 25% would have given me a sample size of 305 in 2008 alone – which would have been more than the anticipated sample size. Unfortunately for the study, there was a dramatic drop in the incidence of malaria in the two seasons. In 2007, I achieved only 25% of my sample size and in 2008 24%. Pooling the data from both malaria seasons and using the lower sample size of 130 occasioned by the dropping of the CQ-SP treatment arm, I was only able to achieve 74% of the expected minimum sample size. The consequence of this was a restriction on the range of sub-analysis that could be conducted.

Another situation which affected the study but which was beyond my control was the change of antimalarial drug policy in The Gambia during the second year of the study. The initial 2x2 design of the study was to enable the investigation of the long term effect of treating a child with either CQ-SP or ACT on macrophageal iron loading. This particular investigation was important to this study because I had hypothesized that part of the mechanisms through which chloroquine will enhance erythropoietic response was by inhibiting macrophageal iron loading. The removal of chloroquine as a first line antimalarial in The Gambia necessitated dropping one of the treatment arms of the study and a modification to the study protocol in the second year of the study. This reduced the number of children on whom the anti-macrophageal iron loading effect of chloroquine could be assessed.

As has already been discussed in this chapter, this study was designed to test the potential use of the anti-inflammatory and immune-modulatory actions of chloroquine on bone marrow response during malaria. But in implementing the protocol, 5mg chloroquine base /kg/week was used which is essentially a prophylactic dose rather than the 3-4mg/kg/day usually used for treatment of inflammatory conditions. Therefore the chloroquine dose used was considerably below the anti-inflammatory dose. The reason for choosing this low dose has already been discussed. It was considered that it was better to err on the side of

caution. It is therefore possible that the low chloroquine dosage might have affected the Hb response.

The potential impact of the high prevalence of iron deficiency in this study has already been discussed. The level of iron deficiency that was found in this study was not anticipated. Since the hypothesis was based primarily on the ability of chloroquine to modulate iron flux in the body, limited quantity of total body iron will limit the effect of chloroquine on body iron mobilisation; and thus could contribute to the null effect found in this study.

As discussed earlier in this chapter, the intervals between the neopterin measurements might have affected our ability to detect differences in macrophageal activation between the chloroquine and placebo arms. This time frame was chosen initially because I was interested in the long term effects of chloroquine on Hb response and my hypotheses depended on evidence of ongoing inflammation for which I found little evidence. Another reason for choosing the longer measurement intervals was because I expected to find children with ongoing sub-microscopic parasitaemia as chloroquine resistance was rapidly increasing. In the presence of sub-microscopic parasitaemia, prolonged chloroquine administration might help in suppressing the continued inflammatory response; but with the parasitaemia completely cleared such benefit might not exist. In this study, the incidence of sub-microscopic parasitaemia was 15% (about 14 children).

In this study, a linear relationship between macrophageal iron acquisition and release on the one hand and Hb response on the other hand was assumed. However, as has been shown in Chapter two (iron metabolism) iron flux in the body is complex and dependent on a number of factors. Therefore, the apparent lack of effect of chloroquine in this study does not preclude an effect on macrophageal iron flux. To answer this question satisfactorily, it will be important to try and measure macrophageal iron directly or to use haemazoin as a proxy measurement.

8.5 Suggestions for future research

1) The relatively large, but insignificant, differences in Hb responses between children treated with CQ-SP and those treated with ACT (see Table 18) merits further investigation. The failure to achieve significance

could have resulted from the small sample size (31) involved in the analysis.

2) The larger initial fall in the CQ-SP group also merits further exploration as this could have clinical implications on the use of CQ-SP as an adjuvant anti-inflammatory agent in the management of children with malaria.

3) The apparent lack of effect of chloroquine on post-malaria Hb recovery in this study should not discourage further probing into its possible role in management of malaria anaemia. A subsequent study along these lines could study a smaller number of participants with more intense investigations, particularly within the first week of diagnosis. It might also be unnecessary to follow-up the participants for 90 days. As shown in this study, by day 30, all the associated malaria-induced inflammation had resolved, suggesting that follow-up for 30 days is adequate for investigating this relationship. In addition, conducting more investigations of the anti-inflammatory effects of CQ during the first week of diagnosis is likely to yield more information than doing so at a later period.

4) As highlighted in sections 8.2 and 8.4, the use of low dose chloroquine might have affected the ability to demonstrate an effect on Hb. This choice was necessary in the absence of safety data on the use of higher chloroquine dose in children with malaria anaemia. It may therefore be necessary to design a much smaller study with more detailed endpoints to assess the safety of a higher dose of chloroquine in children with malaria anaemia, and also its effect on monocyte iron. Such study could combine both *in vitro* and *in vivo* designs comparing clinical/haematological parameters with some mechanistic studies. In particular, direct measurement of macrophageal iron flux in the presence of chloroquine will be desirable.

8.6 Conclusions

Weekly chloroquine administered at a dose of 5mg/kg for three months to children with mild malaria anaemia resulted in no improvement in erythropoietic recovery compared to placebo. I consider that this dose of chloroquine was too low to for anti-inflammatory action. Children with higher Hbs at diagnosis, three years or older, higher HAZ score and without iron deficiency had better Hb

recovery post-malaria. Similar to other reports in the literature, urinary neopterin and IL-10 are both elevated during acute malaria. The role of iron deficiency in erythropoietic recovery post malaria noted in this study suggests potential areas for investigation of the clinical value in administering both chloroquine and iron supplementation to children with malaria anaemia residing in areas of high iron deficiency and malaria burden.

9 Appendices

9.1 Appendix 1: Study Case Record Forms

9.1.1 Morbidity questionnaire

CQ-PMA Form 01

Chloroquine-Post Malaria Anaemia Study

Clinical Questionnaire (Clinquest)

Child's Name:_____

West Kiang No. |__|__|__|__|__|__|__|__|__|__| **Study Day:** |__|__|

Date of Birth:|__|__|__|__|__|__|__|__| **Age (Mth):** |__|__|

Study Date: |__|__|__|__|__|__|__|__| **Village:** _____

Mother's Name:_____ **Father's Name:**_____

Interviewer's name: _____

Received any drugs during this illness? **Yes** |___| **No** |___| **Specify** _____

Clinical Assessment

Symptoms	Yes	No	Comments
Fever			
Fresh cold			
Diarrhoea			
Vomiting			
Excessive crying			
Fast or difficult breathing			
Cough			
History of Convulsion			
Ear problems			
Pale			
Unconscious			
Very weak/cannot stand			
Unable to drink or breastfeed			

Temp (°C) |__|__| : |__| **Heart rate (/min)** |__|__|__|

Resp rate (/min) |__|__|

Malaria dipstick: Positive |___| **Negative** |___| **Not done** |___|

Diagnosis	Yes	No	Comments
Malaria			
Pneumonia			
Coryza			
Acute watery diarrhoea			
Dysentery			
Skin disorders			
Unknown fever			
Others (Please specify)			

9.1.2 Laboratory record form

CQ-PMA Form 02

Chloroquine-Post Malaria Anaemia Study

Laboratory Record Form

Child's Name:_____ **West Kiang No.** |___|___|___|___|___|___|___|___|___|___|

Date of Birth:|___|___|___|___|___|___|___|___| **Study Day:** |___|___|

Village: _____ **Study Date:** |__|__|__|__|__|__|__|__|

Mother's Name:_____**Father's Name:**_____

Malaria

Giemsa Stain: |___|___|___|___|___|___|___|___|

Haematology

ZnPP: |___|___|___|___|**mmol/l** **HB:** |___|___|___|**g/L**

Urine

Urinalysis (for day 0 only)

	Positive	Negative
Protein		
Glucose		
Urobilinogen		
Blood		
Ketones		
Bilirubin		
Leucocytes		
Nitrite		

9.1.3 Anthropometry record form

CQ-PMA Form 03

Chloroquine-Post Malaria Anaemia Study

Anthropometry

Child's Name:_____

Date of Birth:|__|__|__|__|__|__|__|__| **Age (Mth):** |__|__|

Village: _____

Study Date: |__|__|__|__|__|__|__|__| **Sex M/F** |__|

West Kiang No. |__|__|__|__|__|__|__|__|__|__|__|__|

Mother's Name:_____ **Father's Name:**_____

Interviewer's name: _____

Anthropometry

Weight1 (Kg): |__|__|.|__|__| **Length1 (cm):** |__|__|__|.|__|

Weight2 (Kg): |__|__|.|__|__| **Length2 (cm):** |__|__|__|.|__|

Weight3 (Kg): |__|__|.|__|__| **Length3 (cm):** |__|__|__|.|__|

MUAC1 (cm): |__|__|.|__| **SFT1 (mm):** |__|__|.|__|

MUAC2 (cm): |__|__|.|__| **SFT2 (mm):** |__|__|.|__|

MUAC3 (cm): |__|__|.|__| **SFT3 (mm):** |__|__|.|__|

Any comments? _____

9.1.4 Food frequency questionnaire

CQ-PMA Form 04

Chloroquine-Post Malaria Anaemia Study Time started:

Food Frequency Questionnaire

Date of interview [_ _/_ _/_ _]

Interviewer's Name .....

1. **West Kiang Number** [_ _ _ _ _ _]

3. **Child's Name**: .....

4. Informant's Name: .....

5. Informant's relationship to the child: .....

6. Who normally feeds the child? .....

7. **Is your child currently being breastfed?** Yes / No

8. **How much water would you say your child normally drinks in one day** (ESTIMATE AS NUMBER OF BIG OR SMALL CUPS) [_ _ _ _ _ _ _ _ _ _ _ _]

9. Please tell us how many times in one week that your child eats each of the following foods

	Every day		More than 4 times a week		Less than 4 times a week		About once in one week		Additional comments
	Yes	No	Yes	No	Yes	No	Yes	No	
Sour Milk									
Processed milk (powder or liquid)									
Baobab juice (sita nono/iso)									
Baobab fruit added to porridge									
Fish									
Domoda made from meat or fish									
Rice (benachin)									
Janindo made from meat or fish									
Tia nyankatango (Mbahal)									
Porridge (Mono) made from maize (tubanyo)									
Porridge (Mono) made from millet (suno)									
Porridge (Mono) made from findi									

9.1.5 Drug record form for 2007

CQ-PMA Form 05

Chloroquine-Post Malaria Anaemia Study

Drug Record Form 2007

Child's Name:_____

Date of Birth:|__|__|__|__|__|__|__|__| **Age (Mth):** |__|__|

Village: _____

Study Date: |__|__|__|__|__|__|__|__|

West Kiang No. |__|__|__|__|__|__|__|__|__|__|

Mother's Name:_____ **Father's Name:**_____

Child's Weight (Day 0): |__|__|__|__|__| **Child's Weight (Day 10)** |__|__|__|__|__|

Date	Study Day	Drug code	Dosage (mls)	Signature	Comments
	Day 0				
	Day 1				
	Day 2				
	Day 10				
	Day 17				
	Day 24				
	Day 31				
	Day 38				
	Day 45				
	Day 52				
	Day 59				
	Day 66				
	Day73				
	Day 80				
	Day 87				

Note: Children were treated with either CQ-SP or ACT on days 0-2 (open randomisation), and then subsequently randomised to either weekly chloroquine or weekly placebo

9.1.6 Drug record form for 2008

CQ-PMA Form 05

Chloroquine-Post Malaria Anaemia Study

Drug Record Form 2008

Child's Name:_____

Date of Birth:|__|__|__|__|__|__|__|__| **Age (Mth):** |__|__|

Village: _____

Study Date: |__|__|__|__|__|__|__|__|

West Kiang No. |__|__|__|__|__|__|__|__|__|__|

Mother's Name:_____ **Father's Name:**_____

Child's Weight : |__|__|__|__|__|

Date	Study Day	Drug code	Dosage (mls)	Signature	Comments
	Day 10				
	Day 17				
	Day 24				
	Day 31				
	Day 38				
	Day 45				
	Day 52				
	Day 59				
	Day 66				
	Day73				
	Day 80				
	Day 87				

Note: All children were treated with ACT on days 0-2 (hence removed from this form), and then subsequently randomised to either weekly chloroquine or weekly placebo

9.1.7 Adverse event form

CQ-PMA Form 06

Chloroquine-Post Malaria Anaemia Study

Adverse Event Form

Child's Name:_____

Mother's Name:_____ **Father's Name:**_____

West Kiang No. |__|__|__|__|__|__|__|__|__|__|

Study Date: |__|__|__|__|__|__|__|__| **Week:** |__|__|

Interviewer's name: _____

Ask whether the child has had any of the following in the past one week

Symptoms	Yes	No	Comments
Itching			
Nausea (feeling like vomiting)			
Headache			
Vomiting			
Diarrhoea			
Skin rash			
Falling off of hair			
Difficulty with hearing			
History of Convulsion			
Eye problems			
Poor appetite			

If yes to any of the above, grade: 1 |__| 2 |__| 3 |__| 4 |__|

Relationship to investigational product:

Not related 1 |__|

Unlikely 2 |__|

Possible 3 |__|

Probable 4 |__|

Definite 5 |__|

9.1.8 Village Assistants' temperature monitoring chart

CQ-PMA VAMT

Chloroquine-Post Malaria Anaemia Study

Village Assistants' Monitored Temperature (VAMT)

Village: _____

Month: _____

S/no	Name	WKN	Week 1		Week 2		Week 3		Week 4	
			1	2	1	2	1	2	1	2

9.1.9 Exclusions record form

Chloroquine-Post Malaria Anaemia Study

Exclusions Record Form

IndividualID	FirstName	LastName	Sex	Age	MotherName	FatherName	Reasons for exclusion

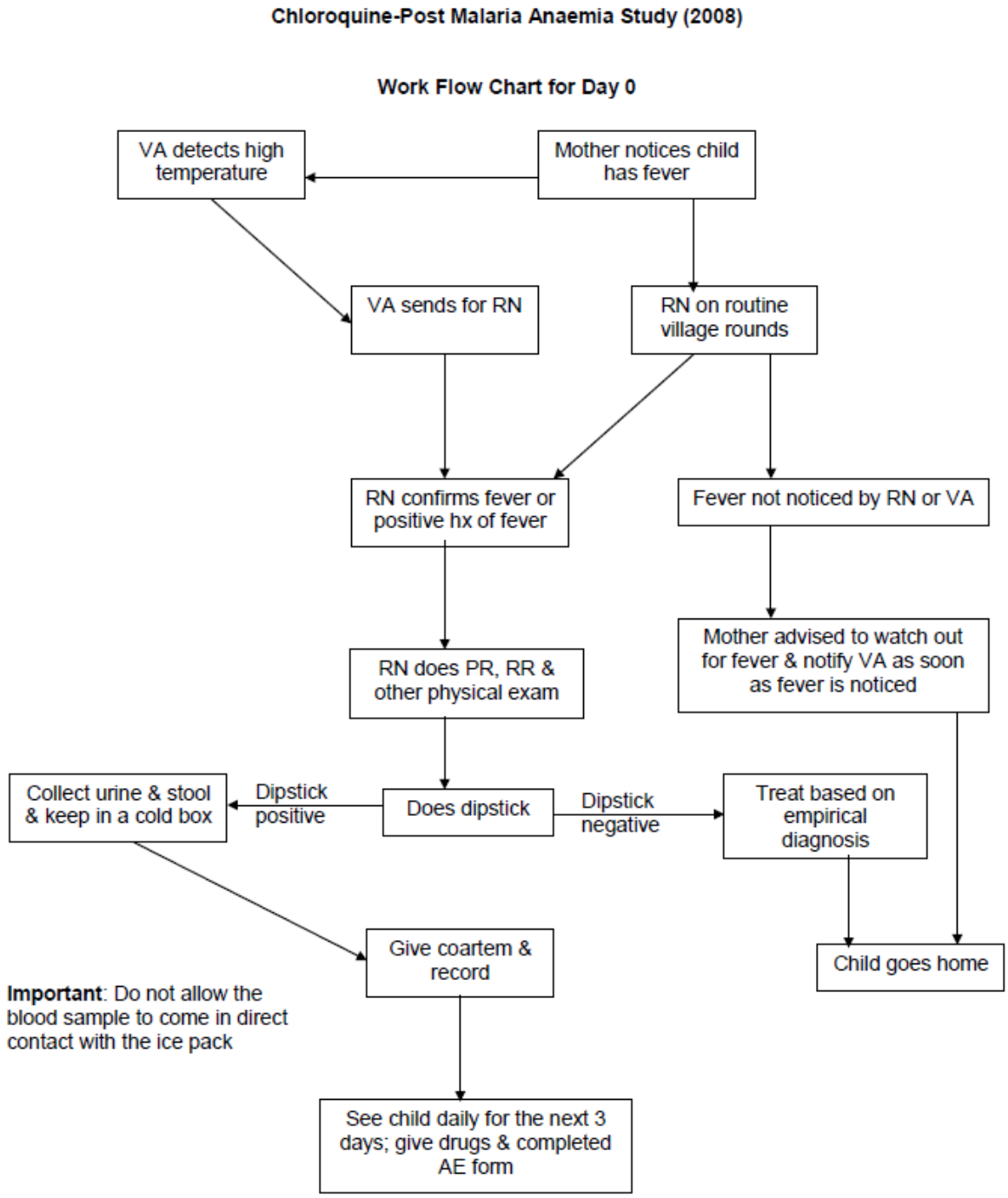
Reasons for exclusion:

Low WHZ score	1
Other medical conditions	2
Refused consent	3
Low Hb at day 3	4
Took antimalarials	5
Left village	6
Died	7

Low parasitaemia	8
Early treatment failure	9
Withdrew	10
High Day 3 Hb	11
Very low Day 3 Hb	12
Second malaria episode	13

9.2 Appendix 2: Work flow charts

9.2.1 Day 0 work procedures

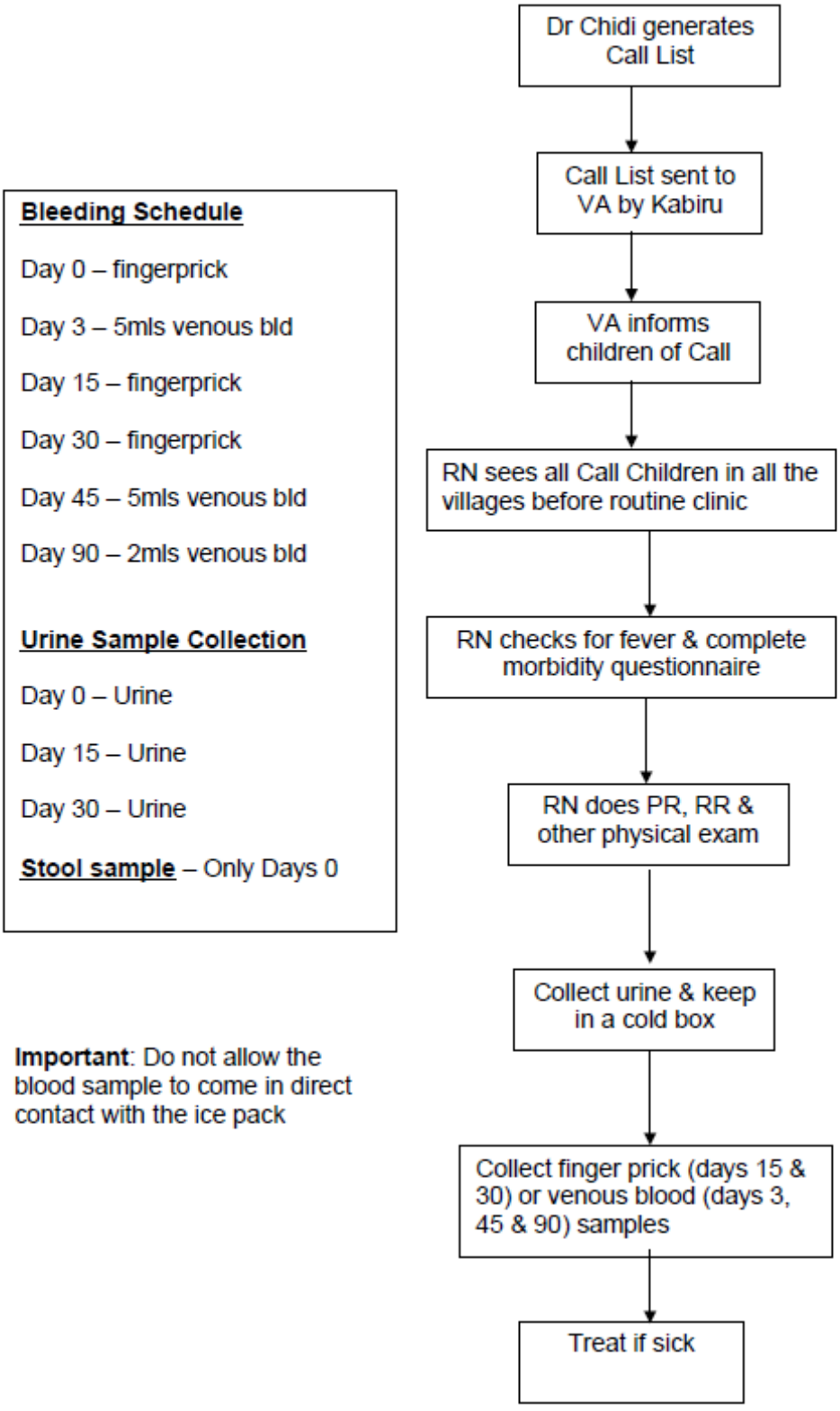


Notes: VA = Village Assistant; RN = Resident Nurse; PR = pulse rate; RR = respiratory rate; AE = adverse event

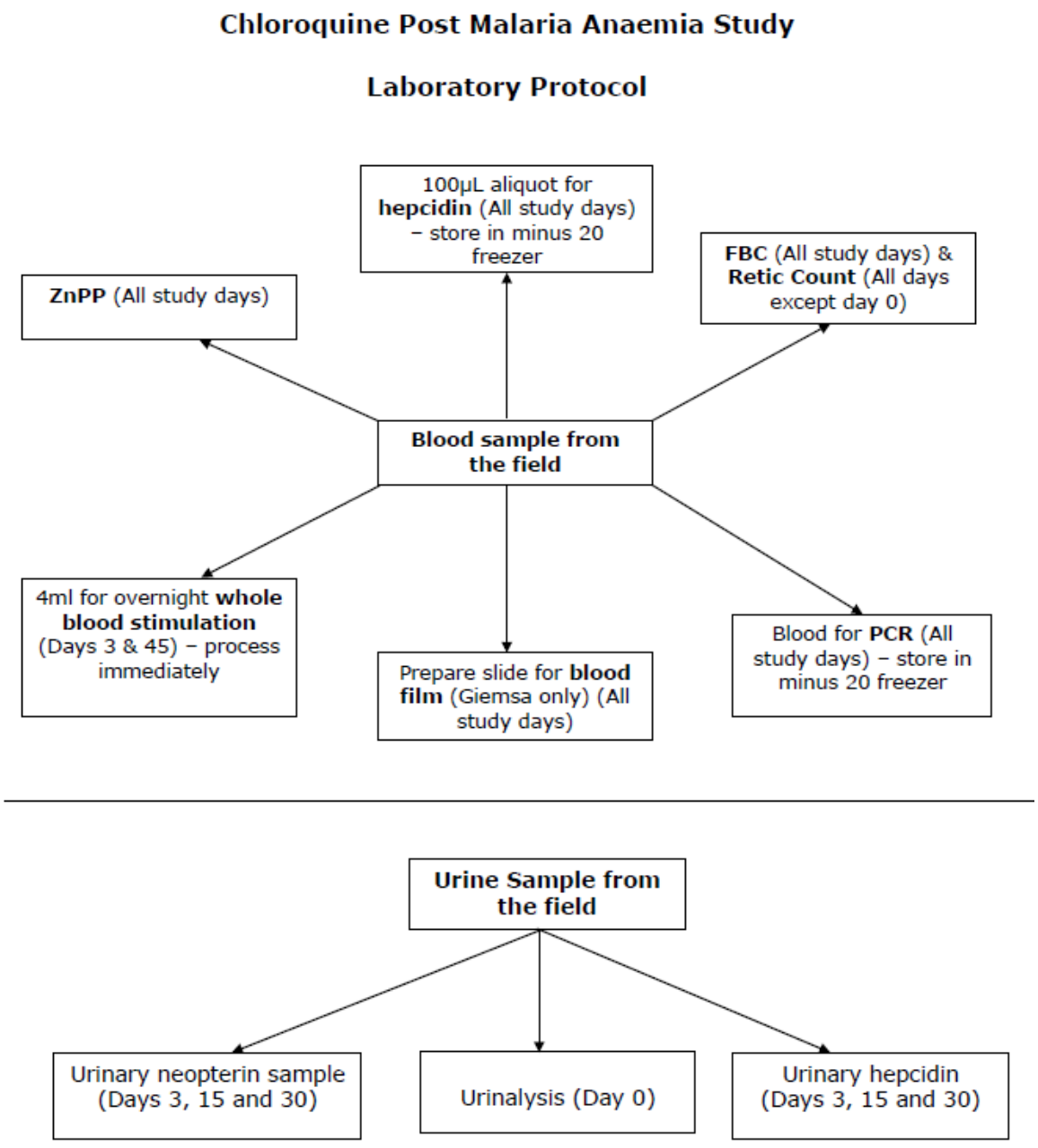
9.2.2 Work flow chart for Call days

Chloroquine-Post Malaria Anaemia Study (2008)

Work Flow Chart for Call Days



9.2.3 Laboratory protocol



9.3 Appendix 3: SOP for DNA Extraction

Standard Operating Procedure (SOP) for DNA Extraction from whole Blood using QIAamp DNA Mini Kit

Before Starting:

- Be sure to put on appropriate lab coats and gloves before starting extraction.
- Decide on number of samples to process at a time, (preferably even numbers) remove from storage (fridge, cooler box or freezers) and equilibrate to room temperature (RT).
- Remove appropriate number of tubes of RNase A from the fridge (stored at 4^0C) and Protease solution from –20^0C freezer and thaw on ice (stored in 400uL aliquots, enough for 20 samples).
- Set heating block or water bath to 56^0C.
- Ensure that buffer AW1 and AW2 have been prepared appropriately (should be marked on the bottle cap with date opened).
- Mix buffer AL properly by shaking before use
- Select a QIAamp spin column for each sample and label with sample ID
- Label 4 sets of DNAase/RNAase-free 1.5mL eppendorf tubes, snip off the caps on the 2nd and 3rd sets but leave covered, label the 4th set with the Study ID, Date and Subject study number copying from the label on the sample container (for storing the DNA).
- All pipetting should be done with aerosol barrier tips preferably.
- Do a quick spin before opening the tubes after each mixing or vortexing step to remove liquid droplets from the inside of the lids.
- Use a different tip for each pipetting operation.
- Ensure there is autoclaved 1X PBS solution ready.
- Be careful not to wet the rim when pipetting into the spin column.
- **Discard wastes into brown waste bottles not into Vikron solution!**

DNA Extraction

1. Pipette 20uL of Protease solution into each of the 1st set of labelled tubes.
2. Mix the blood sample by pipetting up and down a few times and then add 100uL of the blood to the labelled tubes followed by 100uL of 1X PBS solution and mix thoroughly by pipetting up and down.
3. Add 20uL of RNase A to each tube.
4. Add 200uL of buffer AL to each tube and mix by pulse vortexing for 15s (**Important:** Ensure mixture becomes homogenous).

5. Incubate in heating block at 56^{0}C for 30 minutes, centrifuge briefly to remove drops from inside of lid.
6. Add 200uL of ethanol (96-100%) to each sample, mix by vortexing for 15s and centrifuge briefly to remove drops from the inside of the lid.
7. Place the labelled QIAamp spin column in a 2mL collection tube and carefully apply the mixture to the column without wetting the rim. Close the cap and centrifuge at 8000 rpm for 1 min.
8. Place the spin column in a clean collection tube and discard the flow through.
9. Carefully open the spin column and add 500uL of buffer AW1, close the cap and centrifuge at 8000 rpm for 1 min.
10. Place the spin column in a clean collection tube and discard the flow through.
11. Carefully open the spin column and add 500uL of buffer AW2, close the cap and centrifuge at 14000 rpm for 3 min.
12. Place the spin column in the prepared 1.5mL tubes (2nd set) and discard the flow through.
13. Centrifuge the spin column at 14000 rpm for 1 min.
14. Place the spin column in the 3rd set of 1.5mL (DNAase/RNAase-free) tubes, carefully open the spin column and add 50uL of buffer AE ensure the buffer is applied directly to the filter, close and incubate at room temperature for 10 min.
15. Centrifuge the spin column at 8000 rpm for 1 min and transfer the flow-through (DNA) into the 4th set of labelled 1.5mL tube.
16. Store DNA samples at -80^{0}C or –20^{0}C.

9.4 Appendix 4: Protocol for Overnight Whole Blood Culture

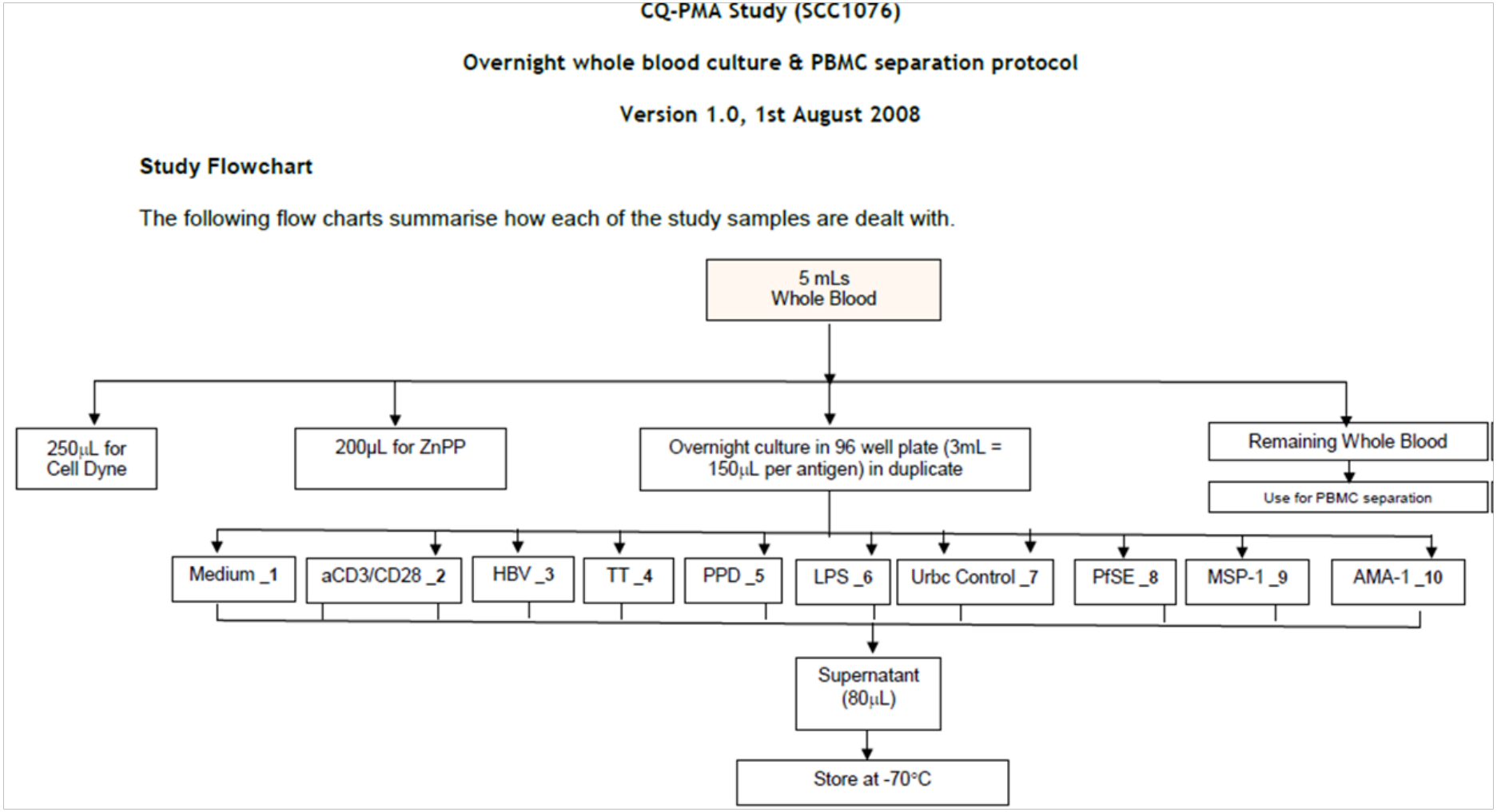
CQ-PMA Study (SCC1076)

Overnight whole blood culture & PBMC separation protocol

Version 1.0, 1st August 2008

Study Flowchart

The following flow charts summarise how each of the study samples are dealt with.



2. Labelling code for donor CQ001 for days 3 & 45 bleed: Study ID = CQ001; day of bleed = 03 or 45; antigen code = 1-10; duration of culture = 1 or 3 (last figure).

Treatment	Sample / culture condition	Present in tube	Code	Study Day
Overnight culture	Medium	day 1 culture supernatant	CQ001_03_1_1	3
Overnight culture	Medium	day 3 culture supernatant	CQ001_03_1_3	3
Overnight culture	Medium	day 1 culture supernatant	CQ001_45_1_1	45
Overnight culture	Medium	day 3 culture supernatant	CQ001_45_1_3	45
Overnight culture	anti-CD3/CD28	day 1 culture supernatant	CQ001_03_2_1	3
Overnight culture	anti-CD3/CD28	day 3 culture supernatant	CQ001_03_2_3	3
Overnight culture	anti-CD3/CD28	day 1 culture supernatant	CQ001_45_2_1	45
Overnight culture	anti-CD3/CD28	day 3 culture supernatant	CQ001_45_2_3	45
Overnight culture	TT	day 1 culture supernatant	CQ001_03_4_1	3
Overnight culture	TT	day 3 culture supernatant	CQ001_03_4_3	3
Overnight culture	TT	day 1 culture supernatant	CQ001_45_4_1	45
Overnight culture	TT	day 3 culture supernatant	CQ001_45_4_3	45
Overnight culture	PPD	day 1 culture supernatant	CQ001_03_5_1	3
Overnight culture	PPD	day 3 culture supernatant	CQ001_03_5_3	3
Overnight culture	PPD	day 1 culture supernatant	CQ001_45_5_1	45
Overnight culture	PPD	day 3 culture supernatant	CQ001_45_5_3	45
Overnight culture	LPS	day 1 culture supernatant	CQ001_03_6_1	3
Overnight culture	LPS	day 3 culture supernatant	CQ001_03_6_3	3
Overnight culture	LPS	day 1 culture supernatant	CQ001_45_6_1	45
Overnight culture	LPS	day 3 culture supernatant	CQ001_45_6_3	45
Overnight culture	Urbc	day 1 culture supernatant	CQ001_03_7_1	3
Overnight culture	Urbc	day 3 culture supernatant	CQ001_03_7_3	3
Overnight culture	Urbc	day 1 culture supernatant	CQ001_45_7_1	45
Overnight culture	Urbc	day 3 culture supernatant	CQ001_45_7_3	45
Overnight culture	PfSE	day 1 culture supernatant	CQ001_03_8_1	3
Overnight culture	PfSE	day 3 culture supernatant	CQ001_03_8_3	3
Overnight culture	PfSE	day 1 culture supernatant	CQ001_45_8_1	45
Overnight culture	PfSE	day 3 culture supernatant	CQ001_45_8_3	45
Overnight culture	MSP_1	day 1 culture supernatant	CQ001_03_9_1	3
Overnight culture	MSP_1	day 3 culture supernatant	CQ001_03_9_3	3
Overnight culture	MSP_1	day 1 culture supernatant	CQ001_45_9_1	45
Overnight culture	MSP_1	day 3 culture supernatant	CQ001_45_9_3	45
Overnight culture	AMA_1	day 1 culture supernatant	CQ001_03_10_1	3
Overnight culture	AMA_1	day 3 culture supernatant	CQ001_03_10_3	3
Overnight culture	AMA_1	day 1 culture supernatant	CQ001_45_10_1	45
Overnight culture	AMA_1	day 3 culture supernatant	CQ001_45_10_3	45
Ex vivo	Whole blood	Plasma	CQ001_03_P	3
Ex vivo	Whole blood	Plasma	CQ001_45_P	45

3. Blood Handling on arrival

The blood sample is divided immediately on arrival in the laboratory as below.

- 250µL to analyse FBC using the Cell Dyne.
- 3mLs to set up overnight cultures with 9 conditions (150µL/well in a 96 well plate in duplicate),
- Remaining blood to be spun for plasma and stored.

4. Overnight whole blood cultures

- Label a 96 well plate well with the sample number and the antigens to be added according to the prepared template

- For each donor sample, dispense 150µl blood into (20) wells in a 96 well plate (2 wells per stimulus and two unstimulated wells).

- Add the stimuli to the appropriate wells:

- Seal plate and incubate at 37°C, 5% CO_2

- The following morning (starting 9am) harvest supernatants from the day 1 wells

- On the third day of culture, harvest supernatants from the day 3 wells

- Take 75µL of culture supernatant and place into pre-labelled tubes. Store at -20°C and record location on lab record sheet

9.5 Appendix 5: Informed consent

9.5.1 Information sheet

MRC Keneba
MRC Laboratories
P.O. Box 273
Banjul, The Gambia
Phone: +2205720329, +2205720330
Fax: +2205720323

Chloroquine as a therapeutic option for mild post malaria anaemia

SCC 1076 Version 2 (July 2008)

Information sheet
(To be translated by the fieldworker into a language understood by the child and parent)

Purpose of the study
We are inviting your child to take part in a research study looking at the treatment of anaemia that often follows infection with malaria. As you know, malaria is still a major problem in The Gambia, especially in young children. One of the reasons why malaria kills a lot of children is because children who get malaria often develop anaemia. Although we currently use iron to treat malaria, this may not be the most effective treatment for children with less severe forms of anaemia. The aim of this study is to see whether chloroquine, which is presently used to treat malaria in The Gambia, can also be used to treat children with mild anaemia from malaria.

What does the study involve?
If you decide that your child can participate in this study, we will firstly register him/her into the study and then take some simple measurements including weight and height. Your child will also be seen by a study nurse to check their general well being and to see if there are any reasons why he or she may not be fit to join in the study. Once enrolled, we will then give him/her a study card and then from the middle of July 2008, the village assistant in charge of this village will visit your child to take his/her temperature twice a week. The level of involvement of your child in the study will then depend on whether or not they develop malaria during the course of the next 5 months. Children who do not develop malaria will simply be seen twice every week to have their temperature measured, until the study finishes in March, 2009.

If at any time your child is seen to have high temperature, a nurse from the MRC will come and see the child. The nurse will then take a finger prick blood sample and use a simple test to check for malaria. If this test suggests that your child has malaria, the nurse will collect a fingerprick sample of blood from the child and begin treatment for malaria. The blood sample collected will then be taken to MRC Keneba where the sample will be further tested for malaria and other blood measurements. We will also collect urine and stool from the child for tests. After 3 days of treatment, your child will be reviewed to be sure that the child is responding to treatment. This will involve collecting 5mls of blood (about one tablespoon) to further test for malaria and other blood measurements. We will also collect urine from the child. The child will then be seen again on the day 15 during which we will collect another finger prick blood sample. to check for malaria and anaemia. If your child is seen to have mild anaemia, they will then be placed on weekly treatment and studied for a further three months.

We will assign your child to two types of weekly treatment. One of these weekly treatments will be chloroquine, and the other one will look like chloroquine but will not contain chloroquine. You will not know which drug is chloroquine, and which one is not; the nurses and the doctors will also not know. This is important so that we will be sure that every child in the study has equal chance of getting chloroquine. After this we will see your child 3 more times (two week later and then once every month for two months). These visits are to allow us to monitor your child's progress. At each visit, we will ask you some questions about the child's health, and then collect some blood samples. If your child does develop malaria during the study period the total amount of blood we will collect over a three month period will only add up to about 12 ml – which is about 2 teaspoons worth.

Do we have to take part in the study?

No. It is not compulsory for you to take part in this study. It is up to you to decide whether you want your child to take part or not. But if you decide to take part, we will ask you to sign a form allowing your child to participate in the study. If after you have started the study, you change your mind, you are free to withdraw without giving reason. A decision to withdraw from the study at any time will not affect the standard of care your child will receive from MRC clinic in Keneba.

Are there any risks or disadvantages of taking part in this study?

There are no known disadvantages or risks for talking part in this study. All the drugs we will use are approved for use during malaria.

Are there any possible benefits of taking part in this study?

Yes there are benefits from joining the study. The major benefit is that your child will be under continuous observation for malaria and should he or she get malaria, we will immediately investigate and treat him/her. We will also follow him/her closely for three months to be sure that the malaria has been completely cleared. We will also treat any other sickness that the child might have during the study. The other benefit is that your child will be helping us to find out how we can help other children that get anaemia from malaria in future.

Will my child's participation in this study be kept confidential?

All information about you or your child collected for this study will be kept strictly confidential. Any information which we will have to give to others not directly connected to this study will have your child's name and address removed so that he or she will not be recognised from it.

What if I have complaints or questions?

If you have any complaints or questions about any aspects of the study, you can contact the person in charge of the study, Dr. Chidi V. Nweneka at the Keneba MRC (Phone 9957634) or the Head of Keneba Field Station, Dr. Sophie Moore (Phone 9963991).

Thank you.

9.5.2 Informed consent signature form

MRC Keneba
MRC Laboratories
P.O. Box 273
Banjul, The Gambia
Phone: +2205720329, +2205720330
Fax: +2205720323

Chloroquine as a therapeutic option for mild post malaria anaemia

SCC 1076 Version 2 (July 2008)

Consent Form
To be translated by the fieldworker into a language understood by the child and parent

Purpose of the study
We are inviting your child to take part in a research study looking at the treatment of the anaemia that often follows infection with malaria. As you know, malaria is still a major problem in The Gambia, especially in young children. One of the reasons why malaria kills a lot of children is because children who get malaria often develop anaemia. Although we currently use iron to treat malaria, this may not be the most effective treatment for children with less severe forms of anaemia. The aim of this study is to see whether chloroquine, which is presently used to treat malaria in The Gambia, can also be used to treat children with mild anaemia from malaria.

1. I confirm that the information sheet about the above study has been read and explained to me, and that I have had the opportunity to ask questions.
2. I understand that the participation of my child in this study is voluntary, and that we are free to withdraw at any time without giving any reason, and without the care my child receives at MRC clinic in Keneba being affected.
3. I understand that the involvement of my child in the study will depend on whether of not they develop malaria over the next few months.
4. I understand that following treatment for malaria my child will then be randomly allocated to different drugs and that neither I nor the research team will know which child receives which drug until the end of the study.
5. I understand that samples of my child's blood, stool and urine will be collected during the study and that these will be stored by the MRC and used for further analysis.
6. I agree that my child should participate in the above study.

Name of Child	Village	West Kiang No.
Name of Parent/Guardian	Date	Signature
Name of person taking consent	Date	Signature

9.6 Appendix 6: Papers resulting from the thesis

9.6.1 Iron delocalisation paper (Published)

Transactions of the Royal Society of Tropical Medicine and Hygiene 104 (2010) 175–184

Contents lists available at ScienceDirect

Transactions of the Royal Society of Tropical Medicine and Hygiene

journal homepage: http://www.elsevier.com/locate/trstmh

Review

Iron delocalisation in the pathogenesis of malarial anaemia

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ARTICLE INFO

Article history:
Received 26 February 2009
Received in revised form 19 August 2009
Accepted 19 August 2009
Available online 23 September 2009

Keywords:
Malaria
Anaemia
Macrophage
Iron
Ferroportin
Hepcidin

ABSTRACT

There is consensus that the pathophysiology of malaria-associated anaemia is multifactorial, but the precise mechanisms behind many of the haematological changes during malaria remain unclear. In this review, we attempt to build a composite picture of the pathophysiology of malarial anaemia using evidence from experimental, human and animal studies. We propose that cytokine- and hepcidin-mediated iron delocalisation, a principal mechanism in the anaemia of inflammation, plays an important role in the aetiology of malarial anaemia, and can explain some of the clinical and laboratory findings. These mechanisms interact with other aetiological determinants, such as dietary iron and micronutrient supply, helminth load, other infections and genetic variation, in determining the severity and associated features of anaemia. We suggest that iron delocalisation as a mechanism for malarial anaemia could be exploited for the development of alternative therapeutic strategies for post-malaria anaemia.

1. Background

Anaemia constitutes a major public health burden in malaria-endemic areas,[1] with prevalence estimates varying from 30 to 90% in children and pregnant women. Although iron deficiency remains the most common cause of anaemia globally, malaria contributes significantly in endemic areas. Severe malarial anaemia increases the risk of death in children.[2] Every year, severe malarial anaemia occurs 1.4 to 5.7 million times, and kills an estimated 190 000–974 000 children below 5 years of age.[3] In an area of Tanzania where malaria transmission is intense and perennial, malaria accounted for 60% of severe anaemia cases among infants.[4] In a Gambian study, as many as 57% of deaths from malarial anaemia occurred within 4 h of admission, most occurring before blood transfusion could be commenced.[5]

Blood transfusion is the standard management of severe malarial anaemia, but is usually not available in rural clinics, and exposes the child to the risk of transfusion-related infections such as HIV and hepatitis.[6] A better understanding of the pathogenesis of malaria-associated anaemia will improve both its prevention and management.[7]

2. Pathogenesis of malarial anaemia

The pathogenesis of malarial anaemia is multifactorial,[8,9] involving the immune- and non-immune-mediated haemolysis of parasitised and non-parasitised erythrocytes, bone marrow dysfunction, altered cytokine balance, nutritional deficits, and interactions with common haemoglobinopathies and erythrocyte defects such as glucose-6-phosphate dehydrogenase deficiency (Figure 1).

2.1. Non-immune and non-specific immune-mediated clearance of erythrocytes in malaria infection

Plasmodium spp. infection results in cycles of erythrocyte rupture and the release of merozoites. With each of

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0035-9203/$ – see front matter.
doi:10.1016/j.trstmh.2009.08.007

9.6.2 Chloroquine review paper (Submitted to Malaria Journal)

Is there a place for chloroquine in the management of post-malaria anaemia?

Chidi V Nweneka

Nutrition Programme, Medical Research Council Laboratories, The Gambia, P.O. Box 273, Banjul, The Gambia

Email address: cnweneka@mrc.gm

Abstract

Background
Anaemia is one of the commonest causes of malaria associated death. The complex pathogenesis of malaria anaemia and the controversies generated by the perceived negative effects of iron administration on malaria-related morbidity and mortality create an urgent need for alternative interventions to combat malaria associated anaemia.

Presentation of the hypothesis
Plasmodium infection initiates a systemic inflammatory process resulting to anaemia. This process involves increased haemolysis, reduced erythropoiesis and iron delocalization. Persisting systemic inflammation despite successful eradication of parasitaemia prolongs the anaemia and could be potentially fatal. Arresting the inflammatory process could minimize the insult to erythropoietic cells and assist erythropoietic recovery post malaria, thereby reducing malaria associated morbidity and mortality.

Testing the hypothesis
Chloroquine is a lysosomotropic agent with potent anti-inflammatory and immunomodulatory properties. It possesses diverse pharmacological actions against a variety of micro-organisms. Although its role in iron metabolism is still poorly understood, there is compelling evidence that many of the effects of chloroquine on organisms result from its interference with intracellular free iron which deprives the organisms of the iron needed for metabolism. There is also strong evidence of an anti-macrophageal iron-loading effect of chloroquine. In plasmodium infection, these properties of chloroquine could be exploited to minimize the associated inflammation and enhance erythropoietic recovery.

Implications of the hypothesis
Considering the wide range of actions of chloroquine, particularly its anti-inflammatory and immunomodulatory properties, it will be a potential candidate for investigation for a possible role in the management of malaria anaemia

Key Words: Chloroquine, Malaria, inflammation, macrophage, iron

9.7 Appendix 7: Ethical Approval

The Gambia Government / MRC Laboratories Joint
ETHICS COMMITTEE

c/o MRC Laboratories Fajara
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Fax: +220 – 4494498 or 4496 513
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14th May 2007

Dr. Chidi Nweneka
MRC, Keneba

Dear Dr. Nweneka

RE: SCC 1076 Chloroquine as a therapeutic option for mild post malaria anaemia.

Thank you for responding to our recent communication regarding the above project. I note the explanation regarding the plans for participants who develop clinical malaria or significant parasitaemia after randomisation. It is also appreciated that you have given thought to the issue about drug resistance in the context of chloroquine monotherapy; and how this will be tackled in practice. Although there is some residual concern about the latter, I am happy to give our approval for the project to proceed as proposed.

Best wishes

Yours sincerely

Mr. Malcolm Clarke
Chairman, Gambia Government/MRC Joint Ethics Committee

9.8 Appendix 8: Comparison of cytokine responses to stimulation with the different antigens between weekly chloroquine group and weekly placebo group

Table 23: comparison of IL-6 values at day 3 between Chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	-226	-20667, 358	0.3
	Placebo	381	-7444, 2888	
TT	Chloroquine	-1505	-1656, -1252	0.5
	Placebo	-3763	-8811, -1230	
PPD	Chloroquine	-524	-807, -449	0.4
	Placebo	13	-2563, 444	
LPS	Chloroquine	-1263	-1406, -114	0.6
	Placebo	-129	-3480, 2430	
PfSE	Chloroquine	-40	-755, 67	0.4
	Placebo	-89	-2488, -9.08	

Table 24: Comparison of IL-6 values at day 45 between Chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	-199	-5145, -136	0.2
	Placebo	-22	7591, 11770	
TT	Chloroquine	-185	-7439, 26	0.8
	Placebo	-1591	-11038, 485	
PPD	Chloroquine	-52	-212, 35	0.3
	Placebo	-2283	-6229, 1844	
LPS	Chloroquine	-111	-980, -22	0.1
	Placebo	-2878	-11704, -281	
PfSE	Chloroquine	-41	-188, 70	1.0
	Placebo	4.72	-269, 328	

Table 25: Comparison of IL-6 values between days 3 and 45 in the chloroquine group

Antigen	Study Day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	-21073	-34538, -2628	1.0
	45	-157	-5145, 245	
TT	3	-15247	-35824, -1580	1.0
	45	-7439	-120840, -44	
PPD	3	-524	-807, -449	1.0
	45	-52	-212, 35	
LPS	3	-1263	-1406, -114	1.0
	45	-111	-980, -22	
PfSE	3	-40	-755, 67	1.0
	45	-41	-188, 70	

Table 26: Comparison of IL-6 values between days 3 and 45 in the placebo group

Antigen	Study day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	-5155	-13809, 48	1.0
	45	38102	-121, 38102	
TT	3	-3951	-12381, -1230	0.3
	45	-110751	-120840, -96	
PPD	3	13	-2563, 444	0.07‡
	45	-2283	-6229, 1844	
LPS	3	-129	-3480, 2430	1.0
	45	-2878	-11704, -281	
PfSE	3	-89	-2488, -9	0.5
	45	5	-269, 328	

‡ Bonferroni adjusted p value = 0.5

Table 27: comparison of IL-10 values at day 3 between Chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	-561	-1284, 37	0.9
	Placebo	-583	-1120, 264	
TT	Chloroquine	-1447	-2247, -667	0.3
	Placebo	-851	-2509, -189	
PPD	Chloroquine	-858	-1908, -495	0.8
	Placebo	-981	-1321, -469	
LPS	Chloroquine	-1374	-1966, -569	0.004‡
	Placebo	300	100, 624	
PfSE	Chloroquine	436	49, 623	0.9
	Placebo	315	48, 768	

‡ Bonferroni adjusted p value = 0.03

Table 28: comparison of IL-10 values at day 45 between Chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	39	2, 255	0.5
	Placebo	-20	-840, 698	
TT	Chloroquine	-153	-1504, -13	0.2
	Placebo	-58	-214, 153	
PPD	Chloroquine	31	-50, 712	0.5
	Placebo	58	-675, 347	
LPS	Chloroquine	88	-6.99, 624	0.4
	Placebo	469	-19, 2250	
PfSE	Chloroquine	8	-2.4, 99	0.8
	Placebo	8	2.2, 37	

Table 29: Comparison of IL-10 values between days 3 and 45 in the chloroquine group

Antigen	Study Day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	-561	-1284, 37	0.4
	45	39	1.63, 255	
TT	3	-1447	-2247, -667	0.1
	45	-96	-1302, -4.43	
PPD	3	-858	-1908, -495	0.006†
	45	31	-50, 712	
LPS	3	-1374	-1966, -569	0.02
	45	88	-6.99, 624	
PfSE	3	436	49, 623	0.007‡
	45	7.71	-2.42, 99	

† Bonferroni adjusted p-value = 0.04; ‡ Bonferroni adjusted p-value = 0.05

Table 30: Comparison of IL-10 values between days 3 and 45 in the placebo group

Antigen	Study day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	-583	-1120, 264	0.1
	45	-20	-840, 698	
TT	3	-851	-2509, -189	0.007‡
	45	-58	-214, 153	
PPD	3	-981	-1321, -469	0.02†
	45	58	-675, 347	
LPS	3	300	100, 624	0.5
	45	469	-19, 2250	
PfSE	3	315	48, 768	0.2
	45	7.72	2.19, 37	

‡ Bonferroni adjusted p-value = 0.05; † Bonferroni adjusted p-value = 0.1

Table 31: comparison of IL-13 values at day 3 between chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	4751	2117, 9137	0.5
	Placebo	2013	845, 8556	
TT	Chloroquine	205	-18.26, 662	0.2
	Placebo	-9.49	-161, 82	
PPD	Chloroquine	-24	-281, -2.64	0.2
	Placebo	-2.39	-46, 118	
LPS	Chloroquine	-49	-112, 13.74	0.5
	Placebo	2.47	-40, 44	
PfSE	Chloroquine	16.37	0.62, 20.81	0.2
	Placebo	-0.98	-22.72, 34.85	

Table 32: comparison of IL-13 values at day 45 between chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	2309	1835, 4998	0.5
	Placebo	953	373, 3562	
TT	Chloroquine	106.42	-28.42, 139.86	0.2
	Placebo	32.81	-4.52, 106.30	
PPD	Chloroquine	22.40	-62.33, 89.16	0.2
	Placebo	79.43	29.16, 162.99	
LPS	Chloroquine	46.31	-134.62, 131.51	0.5
	Placebo	41.09	5.45, 67.60	
PfSE	Chloroquine	5.67	3.85, 19.58	0.2
	Placebo	5.56	-2.72, 26.02	

Table 33: Comparison of IL-13 values between days 3 and 45 in the chloroquine group

Antigen	Study Day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	4751	2117, 9137	1.0
	45	2309	1835, 4998	
TT	3	205	-18, 662	1.0
	45	106	-28, 140	
PPD	3	-23.92	-280.52, -2.64	0.1
	45	22.40	-62.33, 89.16	
LPS	3	-49.29	-112.32, 13.74	0.5
	45	46.31	-134.62, 131.51	
PfSE	3	16.37	0.62, 20.81	1.0
	45	5.67	3.85, 19.58	

Table 34: Comparison of IL-13 values between days 3 and 45 in the placebo group

Antigen	Study day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	2013	845, 8556	1.0
	45	953	373, 3562	
TT	3	-9.49	-161.19, 81.81	0.8
	45	32.81	-4.52, 106.30	
PPD	3	-2.39	-45.81, 117.8	1.0
	45	79.43	29.16, 162.99	
LPS	3	2.47	-39.63, 44.33	0.4
	45	41.09	5.45, 67.60	
PfSE	3	-0.98	-22.72, 34.85	1.0
	45	5.56	-2.72, 26.02	

Table 35: comparison of IL-17 values at day 3 between chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	762	204, 946	0.2
	Placebo	309	160, 696	
TT	Chloroquine	30.48	-26.81, 119.16	0.2
	Placebo	-6.39	-11.93, 15.99	
PPD	Chloroquine	-10.86	-20.20, -2.65	0.05†
	Placebo	-2.41	-3.67, 6.25	
LPS	Chloroquine	-10.10	-26.35, 6.16	1.0
	Placebo	-6.61	-8.57, 2.66	
PfSE	Chloroquine	5.54	-13.09, 31.92	0.4
	Placebo	1.22	-12.44, 7.38	

† Bonferroni adjusted p-value = 0.4

Table 36: comparison of IL-17 values at day 45 between chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	353.37	204, 1200	0.4
	Placebo	282.36	51.51, 495.11	
TT	Chloroquine	13.35	0.00, 23.37	0.1
	Placebo	0.36	-6.59, 3.84	
PPD	Chloroquine	10.16	-10.20, 20.74	0.7
	Placebo	2.73	-2.56, 12.16	
LPS	Chloroquine	-5.56	-14.63, 3.62	0.5
	Placebo	-3.37	-8.92, 1.01	
PfSE	Chloroquine	-1.87	-25.94, 10.57	0.9
	Placebo	1.11	-5.47, 3.05	

Table 37: Comparison of IL-17 values between days 3 and 45 in the chloroquine group

Antigen	Study Day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	762	204, 946	0.6
	45	353	204, 1200	
TT	3	30.48	-26.81, 119.16	0.4
	45	13.35	0.00, 23.37	
PPD	3	-10.86	-20.20, -2.65	0.1
	45	10.16	-10.20, 20.74	
LPS	3	-10.10	-26.35, 6.16	1.0
	45	-5.56	-14.63, 3.62	
PfSE	3	5.54	-13.09, 31.92	0.03†
	45	-1.87	-25.94, 10.57	

† Bonferroni adjusted p-value = 0.2

Table 38: Comparison of IL-17 values between days 3 and 45 in the placebo group

Antigen	Study day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	309	160, 696	1.0
	45	282	51.51, 495.11	
TT	3	-6.39	-11.93, 15.99	1.0
	45	0.36	-6.59, 3.84	
PPD	3	-2.41	-3.67, 6.25	0.5
	45	2.73	-2.56, 12.16	
LPS	3	-6.61	-8.57, 2.66	0.4
	45	-3.37	-8.92, 1.01	
PfSE	3	1.22	-12.44, 7.38	0.5
	45	1.11	-5.47, 3.05	

Table 39: comparison of TNF-α values at day 3 between chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	-1116	-9103, 3717	0.1
	Placebo	40.75	-1187, 3698	
TT	Chloroquine	-2033	-11251, -1724	0.6
	Placebo	-1980	-14423, -1176	
PPD	Chloroquine	-593	-2420, 3592	0.8
	Placebo	-96	-726, 2871	
LPS	Chloroquine	-1125	-1607, 84	0.3
	Placebo	2892	-4198, 4438	
PfSE	Chloroquine	4.27	-65.65, 33.79	0.07†
	Placebo	-25.52	-284.53, -5.66	

† Bonferroni adjusted p-value = 0.5

Table 40: comparison of TNF-α values at day 45 between chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	568	5.91, 4772	0.9
	Placebo	547	-309, 8683	
TT	Chloroquine	-663	-1152, 25.15	0.9
	Placebo	-351	-2263, 3461	
PPD	Chloroquine	445	75, 2114	1.0
	Placebo	365	-966, 6102	
LPS	Chloroquine	456	201, 7440	0.1
	Placebo	-223	-1048, 730	
PfSE	Chloroquine	27.25	-36.24, 153.7	0.04†
	Placebo	-4.59	-60.14, 6.55	

† Bonferroni adjusted p-value = 0.3

Table 41: Comparison of TNF-α values between days 3 and 45 in the chloroquine group

Antigen	Study Day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	-1116	-9103, 3717	0.2
	45	568	5.91, 4772	
TT	3	-2033	-11251, -1724	0.04†
	45	-663	-1152, 25.15	
PPD	3	-593	-2420, 3593	0.6
	45	445	75, 2114	
LPS	3	-1125	-1607, 85	0.03‡
	45	456	201, 7440	
PfSE	3	4.27	-65.65, 33.79	0.3
	45	27.25	-36.24, 153.7	

† Bonferroni adjusted p-value = 0.3; ‡ Bonferroni adjusted p-value = 0.2

Table 42: Comparison of TNF-α values between days 3 and 45 in the placebo group

Antigen	Study day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	41	-1187, 3698	0.7
	45	547	-309, 8683	
TT	3	-1980	-14423, -1176	0.1
	45	-351	-2263, 3461	
PPD	3	-96	-726, 2871	1.0
	45	365	-966, 6102	
LPS	3	2892	-4198, 4438	1.0
	45	-223	-1048, 730	
PfSE	3	-25.52	-284.53, -5.66	0.3
	45	-4.59	-60.14, 6.55	

Table 43: comparison of IFN-γ values at day 3 between chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	5867	559, 37425	0.9
	Placebo	7140	15, 22628	
TT	Chloroquine	-932	-2138, 222	0.5
	Placebo	-302	-1919, 1040	
PPD	Chloroquine	559	-1087, 1426	0.6
	Placebo	251	-28, 2220	
LPS	Chloroquine	-1470	-2031, -1030	0.06†
	Placebo	45	-237, 644	
PfSE	Chloroquine	541	76, 1833	0.7
	Placebo	454	87, 1668	

† Bonferroni adjusted p-value = 0.4

Table 44: comparison of IFN-γ values at day 45 between chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	1287	645, 11346	0.3
	Placebo	458	107, 5831	
TT	Chloroquine	-93	-325, 46	0.8
	Placebo	105	-1490, 623	
PPD	Chloroquine	279	4.16, 1692	0.02†
	Placebo	2459	818, 8591	
LPS	Chloroquine	24	-148, 424	0.08‡
	Placebo	-418	-1083, 176	
PfSE	Chloroquine	5.14	-41, 478	0.5
	Placebo	179	-9.74, 443	

† Bonferroni adjusted p-value = 0.1; ‡ Bonferroni adjusted p-value = 0.6

Table 45: Comparison of IFN-γ values between days 3 and 45 in the chloroquine group

Antigen	Study Day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	5867	559, 37425	0.1
	45	1287	645, 11346	
TT	3	-932	-2138, 222	0.6
	45	-93	-325, 46	
PPD	3	559	-1087, 1426	0.4
	45	279	4.16, 1692	
LPS	3	-1470	-2031, -1030	0.5
	45	24	-148, 424	
PfSE	3	541	76, 1833	0.1
	45	5.14	-41, 478	

† Bonferroni adjusted p-value = 0.3; ‡ Bonferroni adjusted p-value = 0.2

Table 46: Comparison of IFN-γ values between days 3 and 45 in the placebo group

Antigen	Study day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	7140	15, 22628	0.5
	45	458	107, 5831	
TT	3	-302	-1919, 1040	1.0
	45	105	-1490, 624	
PPD	3	251	-28, 2220	0.1
	45	2459	819, 8591	
LPS	3	45	-237, 644	0.7
	45	-418	-1083, 176	
PfSE	3	454	87, 1668	1.0
	45	179	-9.74, 443	

Table 47: comparison of MIF values at day 3 between chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	-505	-2074, 1998	0.5
	Placebo	-1248	-5268, 728	
TT	Chloroquine	-1029	-2070, 2058	0.5
	Placebo	-1745	-4679, 2099	
PPD	Chloroquine	957	-705, 3577	0.08†
	Placebo	-992	-2950, 1107	
LPS	Chloroquine	-923	-2386, -317	0.08†
	Placebo	660	-1972, 3237	
PfSE	Chloroquine	10	-365, 855	0.2
	Placebo	-199	-3876, 437	

† Bonferroni adjusted p-value = 0.6

Table 48: comparison of MIF values at day 45 between chloroquine group and placebo group

Antigen	Study group	Median cytokine concentration	Inter-quartile range	Mann Whitney P-value
aCD3/aCD28	Chloroquine	-165	-683, 140	0.3
	Placebo	506	-1976, 4870	
TT	Chloroquine	55	-510, 811	0.3
	Placebo	383	-1096, 3315	
PPD	Chloroquine	377	-515, 1706	0.6
	Placebo	-269	-2524, 2777	
LPS	Chloroquine	163	-305, 1857	0.4
	Placebo	-57	-5271, 2623	
PfSE	Chloroquine	-406	-1383, 574	0.7
	Placebo	209	-1789, 881	

Table 49: Comparison of MIF values between days 3 and 45 in the chloroquine group

Antigen	Study Day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	-505	-2074, 1998	0.3
	45	-165	-683, 140	
TT	3	-1029	-2070, 2058	0.1
	45	55	-510, 811	
PPD	3	957	-705, 3577	1.0
	45	377	-515, 1706	
LPS	3	-923	-2386, -317	0.02†
	45	163	-305, 1857	
PfSE	3	10	-365, 855	0.2
	45	-406	-1383, 574	

† Bonferroni adjusted p-value = 0.1

Table 50: Comparison of MIF values between days 3 and 45 in the placebo group

Antigen	Study day	Median cytokine concentration	Inter-quartile range	Wilcoxon Signed Rank Test P-value
aCD3/aCD28	3	-1248	-5268, 728	1.0
	45	506	-1976, 4870	
TT	3	-1745	-4679, 2099	0.1
	45	383	-1096, 3315	
PPD	3	-992	-2950, 1107	0.8
	45	-269	-2524, 2777	
LPS	3	660	-1972, 3237	0.7
	45	-57	-5271, 2623	
PfSE	3	-199	-3876, 435	0.4
	45	209	-1789, 881	

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