BMJ open

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Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2013-002666
Article Type:	Research
Date Submitted by the Author:	31-Jan-2013
Complete List of Authors:	Nweneke, Chidi; MRC Keneba, Cox, Sharon; London School of Hygiene & Tropical Medicine, MRC International Nutrition Group; Muhimbili Wellcome Programme, Doherty, Conor; Royal Hospital for Sick Children, Fulford, Anthony; London School of Hygiene andTropical Medicine, MRC International Nutrition Group Moore, Sophie; London School of Hygiene andTropical Medicine, MRC International Nutrition Group Prentice, Andrew; London School of Hygiene andTropical Medicine, MRC International Nutrition Group; MRC Keneba,
Primary Subject Heading:	Haematology (incl blood transfusion)
Secondary Subject Heading:	Epidemiology, Nutrition and metabolism, Infectious diseases
Keywords:	TROPICAL MEDICINE, Clinical trials < THERAPEUTICS, Anaemia < HAEMATOLOGY

Randomised controlled trial of weekly chloroquine to re-establish normal erythron iron flux and haemoglobin recovery in post-malarial anaemia

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Sources of support: This study was supported by the UK Medical Research Council (MC-A760-5QX00). The funder had no role in the design, analysis or reporting of this trial.

Running head: Chloroquine in post-malarial anaemia

Abbreviations used: ACT, co-artemether; CQ-SP, chloroquine plus sulphadoxine-pyrimethamine; Hb, haemoglobin; MCH, mean cell haemoglobin; MCV, mean cell volume; RCT, randomised controlled trial; SMA, severe malarial anaemia; ZnPP, zinc protoporphyrin.

Trial registration: The clinical trial registration number is NCT00473837 (ClinicalTrials.gov).

Abstract

Objective: To determine if low-dose weekly chloroquine (CQ) therapy improves recovery from malaria-associated anaemia.

Design: A proof of concept randomised clinical trial.

Setting: West Kiang District, Lower River Region, The Gambia

Participants: Children resident in participating communities, aged 12-72 months with uncomplicated malaria identified using active case detection over two consecutive malaria transmission seasons.

Interventions: In 2007 eligible children were randomised to chloroquine-sulphadoxine/pyrimethamine (CQ-SP) or co-artemether (ACT) anti-malarial therapy, and after parasite clearance on Day 3 subsequently re-randomised (double-blind) to weekly low-dose CQ (5mg/kg) or placebo. In 2008 all eligible children were treated with ACT and subsequently randomised to CQ or placebo.

Outcome measures: The primary outcome was change in haemoglobin from baseline (Day 3 of anti-malarial treatment) to Day 90 in the weekly CQ or placebo treatment arms. Secondary outcomes were changes in urinary neopterin as a marker of macrophage activation, markers of erythropoietic response and prevalence of sub-microscopic parasitaemia. Change in haemoglobin in the placebo arm by initial anti-malarial treatment was also assessed.

Results: In 2007 101 children with uncomplicated malaria were randomised to anti-malarial treatment with CQ-SP or ACT and 65 subsequently randomised to weekly CQ or placebo. In 2008 all children received ACT anti-malarial treatment and 31 subsequently randomised to weekly CQ or placebo. Follow up to Day 90 was 96%. There was no effect of weekly CQ *vs.* placebo on change in haemoglobin at Day 90, (CQ: +10.04 g/L [95% CI:6.66/13.42] *vs.* placebo: +7.61 g/L [95% CI:2.88/12.35]). There was also no effect on any of the secondary outcomes assessed, or effect of initial anti-malarial therapy on haemoglobin recovery. Higher Day 90 haemoglobin was independently correlated with older age, not being stunted, higher haemoglobin at Day 0 and adequate iron status at Day 3.

Conclusions: Weekly low-dose CQ after effective anti-malarial treatment is not effective in improving recovery from post-malaria anaemia.

Article Focus

- To determine if 3 months of low-dose weekly chloroquine (CQ) (5mg/kg) therapy after effective treatment of uncomplicated malaria can improve haemoglobin recovery after malaria-associated anaemia in rural African children.
- To test the hypothesis that chloroquine may enhance haematologic recovery after a malaria episode through its anti-inflammatory and immuno-modulatory actions, anti-macrophageal iron loading effect, and anti-malarial effect (despite widespread resistance).

Key Messages

- Weekly low-dose CQ after effective anti-malarial treatment was not effective in improving recovery from mild to moderate post-malaria anaemia.
- Weekly low dose CQ at 5mg/kg had no clear effects on erythropoietic responses as determined by reticulocyte percentage, or immune-mediated inflammation as assessed by urinary neopterin, a marker of macrophage activation.

Strengths and limitations

- The strengths of this study are its randomised design and double blinding of the allocation to weekly CQ or placebo and that few subjects were lost to follow-up. The continuous follow-up and active case detection for all children ensured that malaria infections were detected early and within similar time-frames, plus resurgent or new clinical infections were excluded from the analysis of haemoglobin change. A strict malaria case definition was adhered to and the measurement of sub-microscopic parasitaemia over time allowed the effects of incomplete treatment to be modelled.
- A limitation of the study was the need to recruit participants over 2 malaria transmission seasons and the significantly reduced malaria transmission rate in 2008 which also appeared to affect the severity of malaria in terms of parasite densities at first detection and

haemoglobin concentrations, with significantly lower baseline Hb in 2008. Related to this limitation is the effect on the reduced number of participants recruited in 2008.

- It is possible that the relatively low dose of CQ we used was insufficient to mediate the effects that we were testing for and we were unable to conduct pilot studies of CQ drug dosage and duration to determine the potential best treatment regime to test or of the timings of evaluation of possible mechanisms of effect.
- The change in design in 2008 to all children receiving ACT anti-malarial treatment did not affect the analysis of the primary outcome. The change did, however, limit our ability to determine the effect of initial anti-malaria therapy on haemoglobin response in the placebo group. An effect of anti-malarial treatment CQ on Hb, when in the context of adequate parasite clearance as was observed, might have been expected to occur in the early period of recovery.

Introduction

The precise contribution of malaria to anaemia is difficult to calculate mainly because many anaemia-causing conditions co-exist in children living in malaria-endemic countries. [1] Murphy and Breman [2] estimate the annual incidence of severe malaria anaemia (SMA) to be 1.42 to 5.66 million resulting in between 194,000 to 974,000 deaths in children younger than five years. SMA increases the risk of death in children with malaria [3], many of whom die shortly after admission before blood transfusion can be commenced [4 5] and after discharge in the community [6]. In addition, blood transfusion, the standard treatment for severe malaria anaemia exposes the child to the risk of transfusion-related infections like Human Immunodeficiency Virus (HIV) and hepatitis [7]. Among a nationally representative sample of children aged one to five years in The Gambia in 1999, 76% had a haemoglobin (Hb) less than 11g/dl and 15% had Hb less than 7g/dl [8]. It is likely that most of the cases of moderate to severe anaemia were due in great part to malaria [9-11]. A better understanding of the pathogenesis of malaria anaemia is required to improve its prevention and management.

Multiple immune and non-immune-mediated mechanisms cause anaemia during and after a malaria episode[12] including the destruction of non-parasitized erythrocytes which may account for more than 90% of erythrocyte loss[13 14]. One important component is inflammation-induced iron delocalisation which is characterised by sequestration of iron by the monocyte-macrophage system and hepatocytes, resulting in a state of pseudo-iron deficiency; a state that reverses after the inflammation has subsided[12]. The trans-membrane protein ferroportin is the exclusive route for cellular iron efflux and is responsible for releasing absorbed iron from enterocytes and sequestered iron from macrophages[12 15 16]. Hepcidin, the master-regulator of iron metabolism which responds both to iron status and inflammation[15], blocks ferroportin. Clinically, malaria-induced inflammation has been shown to be associated with a hepcidin-mediated reduction in intestinal absorption of iron. [17] Inflammatory cytokines, particularly IL-6 and IL-1 stimulate hepcidin release[18 19] resulting both in reduced iron absorption of dietary iron and impaired macrophageal release of iron; the latter effect being quantitatively much more significant as macrophageal iron recycling and transport to the

erythron is 25-30 fold greater than the influx of dietary iron. In this way hepcidin is the mediator of the anaemia of chronic disease[15]. TNF-α could also play a role in ferroportin-mediated iron transfer independent of hepcidin[20]. Rapid resolution of malaria-induced inflammation could therefore limit the degree and duration of malaria anaemia by reducing the duration of iron delocalisation.

Chloroquine is a cheap and safe antimalarial with proven anti-inflammatory properties; and has been shown to reduce iron delocalisation *in vitro*[21 22]. We hypothesised that chloroquine may enhance haematologic recovery after a malaria episode through its anti-inflammatory and immuno-modulatory actions, anti-macrophageal iron loading effect, and anti-malarial effect (despite widespread resistance). We designed a proof of concept study to investigate the potential use of chloroquine in the management of mild post malaria anaemia.

Materials and Methods

Study Design and population

This study was a population-based randomised, controlled trial (RCT) in children aged 12 to 72 months conducted over two malaria seasons in 2007 and 2008 in West Kiang District, Lower River Region, The Gambia. The study was initially designed and conducted as a 2x2 randomised placebo-controlled trial in 2007 but had to be changed, due to changes in national malaria treatment guidelines, to a two-arm RCT during the 2nd malaria transmission season in 2008. In the 2x2 phase of the study, children with malaria were initially openly randomised to receive a therapeutic dose regime of chloroquine plus sulphadoxine-pyrimethamine (CQ-SP) or co-artemether (ACT) (Day 0). After treatment, on Day 3, children were subsequently randomised (double blind) to weekly chloroquine or placebo until Day 90 (**Figure 1**). In the two-arm phase, all enrolled children with malaria were treated with ACT and then randomised and followed up as before (**Figure 1**). A combination of active and passive case detection was used to identify children in participating communities with malaria using a standard case definition of clinical malaria (see below).

The climate of this district is typical of sub-Sahelian Africa with a long dry season lasting from November to June and a relatively short rainy season lasting from July to October. Malaria transmission is seasonal, hyperendemic and follows the rainy season, occurring between July and December. The prevalence of children experiencing one or more malaria episodes among a cohort of 1002 children aged between 1-6 years under active malaria surveillance during a population-based study in 2003 was 34% (Doherty et al. Unpublished data). During the study period standard malaria control activities in the district consisted of insecticide treated bednet distribution and case finding and treatment. In 2008 the standard first-line antimalarial treatment regimen for uncomplicated malaria in The Gambia was changed from CQ-SP to co-artemether.

Inclusion & exclusion criteria

Eligibility for malaria surveillance and subsequent possible enrolment into the RCT included all children in the participating communities, aged between 12 and 72 months at the start of the

surveillance period and who were not severely wasted, defined as weight-for-height Z scores less than -3SD.

Inclusion criteria for enrolment included: 1) uncomplicated malaria at Day 0, defined as a history of fever 48 hours prior to presentation or a measured temperature of 37.5°C or more with peripheral parasitaemia (asexual forms of *P. falciparum* ≥500/µL by microscopy) on Day 0 of the study; and 2) for randomisation to the post-malaria intervention, children had to have a Hb between 69g/L - 110g/L on Day 3 with no peripheral parasitaemia.

Children were excluded from the RCT if they were: 1) unable to take oral medications; 2) had features of severe malaria;[1] 3) had a known haemoglobinopathy; 4) were enrolled in another project; 5) had already received antimalarial drugs from other sources outside the project team; or 6) were prescribed other drugs with potential antimalarial or anti-anaemic effects such as co-trimoxazole and haematinics.

Following randomisation to weekly CQ or placebo children were withdrawn from the study if: 1) they developed a second malaria episode or had life-threatening symptoms, more severe disease or serious adverse reactions; 2) left the study area for more than 14 days; 3) received antimalarials from other sources; or 4) Hb fell below 70g/L.

Field and clinical procedures

To effect the surveillance protocol, the study villages were divided into geographically adjacent clusters supervised by a resident study nurse who visited each village morning and evening and provided 24-hour clinical coverage to all enrolled children in their cluster. The active surveillance consisted of twice weekly temperature monitoring of enrolled children by village assistants; while the passive surveillance consisted of mothers referring sick children to the visiting study nurse. Children with fever – reported or measured – were screened for malaria using a rapid diagnostic test (MRDT) (Cortez diagnostics, INC, USA) after clinical evaluation. Children testing negative to the MRDT were treated based on the likely diagnosis from the symptoms and signs. Children testing positive to the MRDT were commenced on antimalarials immediately (either CQ-SP or ACT in 2007,

and ACT in 2008 for three days) and directly observed by the study nurse and managed as appropriate. Finger prick blood samples (days 7, 15, 30 & 70) were collected in an EDTA microtainer while venous blood (2mls – days 0 & 90; and 5mls – days 3 & 45) samples were collected in an EDTA vacutainer and sent to the study laboratory on the same day. Co-morbidities were treated as appropriate, avoiding co-trimoxazole and haematinics. For the subsequent visits after initial malaria diagnosis at Day 0, children were seen by the study nurse and blood samples and clinical data collected as per protocol. Only children who fulfilled the inclusion criteria for advancement to the weekly chloroquine/weekly placebo phase of the study were sampled after Day 3. These children were also followed up on days 15, 30, 45, 70 (except in 2008) and 90.

Randomisation and blinding

The randomisation to malaria treatment group in 2007 (CQ-SP or ACT) was open (non-blinded to trial staff, investigators or participants) and was done by sequential assignment as children were identified. The block randomisation to the post-malaria treatment of weekly CQ or placebo in both 2007 and 2008 was double blinded and was carried out in blocks of eight. The randomisation codes were generated by a staff member independent of the study team and held by the external trial monitor. Treatment codes were labelled A to H and placed in sequentially numbered, opaque sealed envelopes held by the study nurses. Allocation to the treatment was by matching the code in the envelope to a bottle of the intervention labelled with the same code and then labelled with the subject ID.

Interventions

For children openly randomised to malaria treatment at Day 0 with CQ-SP, children received choroquine syrup (Chlormal®, Medreich Sterilab Ltd, Avalahalli, Bangalore, India) consisting of 50mg chloroquine base per 5mls as chloroquine phosphate at a dose of 10mg/kg for 3 days (days 0-2). SP (Micro Laboratories, India) was co-administered as a 525 mg tablet (500mg sulfadoxine and 25mg pyrimethamine) on Day 0 only at a dose of ½ tablet/10kg. Children who were randomised to ACT treatment in 2007 and all children in 2008 received co-artemether (Coartem®) tablets (Beijing

Novartis Pharma Ltd, Beijing China for Novartis Pharma AG, Basle, Switzerland) as blister packets of 6 tablets per card with each tablet containing 20mg artemether and 120mg lumefantrine. The treatment dose comprised a stat dose on Day 0 and then 8 hours later, followed by twice daily doses for the next two days with doses depending on body weight (5 - <15 kg – one tablet/dose; 15 - <25kg – 2 tablets/dose; 25 - <35kg – 3 tablets per dose).

During the second blinded phase of the trial children received either weekly CQ syrup (as above) but at a dose of 5mg/kg or matching placebo syrup (prepared by Royal Victoria Teaching Hospital pharmaceutical laboratory in collaboration with Atlantic Pharmaceuticals Limited, Latrikunda Sabiji, The Gambia) The syrups were in similar amber coloured bottles with matching caps and labels. Doses were administered in the child's home under direct observation by the study nurses who waited for at least 10 minutes after administering the drugs to ensure that they were not vomited.

Primary and secondary outcomes

The primary outcome measure was Hb change from Day 3 post treatment of malaria episode to Day 90 in the weekly CQ and placebo arms.

The secondary outcomes were to compare between the weekly CQ and placebo arms: (1-i) changes in erythropoietic response between Day 3 and Day 30; and (1-ii) between day 3 and day 90; (2) changes in urinary neopterin from Day 3 to Day 30 as a marker of macrophage activation; (3) prevalence of sub-microscopic malaria parasitaemia using PCR at each time point, and; (4) Hb change from Day 3 to Day 90 in the two placebo arms to investigate the effects of initial malaria treatment therapy.

Sample size

During initial study design we used a working assumption of a standard deviation (SD) for change in Hb of 15 g/l[23]. With power 80% and significance level set at 5% we estimated the sample sizes required to detect varying effect sizes for the primary outcome. A sample size of 65 per arm

was estimated to be able to detect an effect size of 7.5g/l. This sample size was estimated to be achievable and the effect size likely be clinically significant.

Laboratory procedures:

Table 1 describes the different laboratory analysis conducted. Zinc protoporphyrin (ZnPP) as a marker of iron status was measured on fresh whole blood using a fluorometer (AVIV Biomedicals, Lakewood, NJ, USA). Full blood count (FBC), haemoglobin and automated reticulocyte count were performed using a Cell Dyne 3700 (Abbott Laboratories. Abbott Park, Illinois, USA). Iron deficiency was defined using 3 parameters; mean cell volume (MCV), mean cell haemoglobin (MCH) and ZnPP. The cut off values for the different parameters employed were: ≥61µmol/mol of Hb for ZnPP [24], ≤73fl for MCV [25] and ≤25pg for MCH [25]. Combining these parameters has been shown to increase their sensitivity and specificity to identify iron deficiency [26]. Malaria parasites were identified in a thick peripheral blood smear stained with Giemsa overnight and read by light microscope with x100 magnification in oil immersion. Parasite DNA extraction and subsequent quantitative PCR amplification were carried out using established protocols [27]. In brief, DNA extraction was done using the QIamp DNA Mini Kit (Qiagen™ Ltd. UK) according to the manufacturer's instructions with minor modifications. Extracted DNA samples were stored at -20°C. Quantification of parasite density was done with a TaqMan assay employing a fluorogenic probe to detect specific parasite products and using an Opticon® 2 Real Time PCR machine and Opticon™ version 3.1 software (Bio-Rad Laboratories, Inc., USA). Primers and probes were synthesized by Applied Biosystems, UK. Urinary neopterin levels were measured using an ELISA technique (BRAHMS Neopterin EIA, B.R.A.H.M.S, Germany). The stored urine was allowed to thaw at room temperature and then diluted 1:100 and the rest of the procedure was according to the manufacturer's instructions.

Statistical analysis:

Summary statistics are presented as means and standard deviations (SD) for normally distributed continuous variables, 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 are presented for log-transformed variables. Two-sided student's t tests were used to compare results between two treatment arms.

Visual inspection of Hb change between baseline at Day 3 and later time points by treatment arm suggested that by Day 30, Hb change in both treatment arms had reached a plateau. Therefore Hb data from Day 30 to Day 90 were pooled for subsequent analysis. This pooled Hb is referred to as 'final Hb'. To fully determine the effect of treatment group on Hb change we used random effects models. Initial malaria treatment group (CQ-SP vs ACT), Hb at recruitment (Day 0 Hb), baseline Hb (Day 3), age in months, year of study, and the child's village were included in models "*a-priori*" and hence adjusted for.

Linear regression was used to investigate further potential predictors of Hb change in the study population. First, 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 to assess confounding. The final regression model consisted of only the variables that maintained a significant association with Hb in the multiple regression models. All analyses were carried out using STATA version 8 (StataCorp, College Station, TX, USA).

Ethical clearance

The Scientific Coordinating Committee of the Medical Research Council (UK), The Gambia, and the MRC/Gambia Government Joint Ethical Committee approved the study (SCC 1076). Written informed consent was obtained from parents/guardians of the children prior to enrolment. The clinical trial registration number is NCT00473837 (ClinicalTrials.gov).

Results

Characteristics of the study population under malaria surveillance

Figure 1 describes the flow of participants and reasons for exclusions in the study. In 2007, 1445 of 1817 (80%) potentially eligible children identified through the Keneba MRC DSS database from 14 villages were included in the malaria surveillance. In 2008, 1220 of the 1413 (86%) identified children from 5 of the original villages were included in the malaria surveillance (smaller villages were excluded as they had contributed few cases in 2007). **Table 2** describes the characteristics of the children placed under surveillance for malaria. The proportion of children with a positive malaria rapid test during surveillance (only first event per child counted) fell from 7.3% (105/1445) in 2007 to 4% (49/1220) in 2008. In the first year of the study, 101 (96%) of the children with a positive malaria rapid test were randomised to CQ-SP or ACT treatment, and 65 (64%) subsequently randomised at Day 3 to weekly chloroquine or placebo, with all children completing follow up until Day 30 and 33/34 completing follow-up until Day 90 in the weekly CQ and 28/31 in the weekly placebo group. In the second year of the study 31 of the 49 (63%) cases with a first positive malaria rapid test were subsequently randomised to weekly CQ or placebo after treatment with ACT, all of whom completed follow up until Day 90 (16 CQ & 15 placebo). After determining a lack of change between Day 30 and Day 90 Hb measurements across all treatment groups it was decided to include the 4 children who were excluded after Day 30 of follow-up due to development of a second malarial episode, in the analysis of "final Hb" by use of Hb at Day 30. Thus 96 children were included in the analysis of the primary and secondary outcomes, except for the analysis of effect of initial malaria treatment within the placebo arms which included 31 children.

Table 3 describes the characteristics of participants randomised to weekly chloroquine and weekly placebo for both years combined. Demographic and anthropometric characteristics were similar between the groups as was baseline Hb at Day 3. The prevalence of iron deficiency measured at baseline was 43.1% (28/65) in 2007 and 58.1% (18/31) in 2008 with a similar prevalence between the CQ/placebo groups within each year (data not shown) and in both years combined. However

parasite density was lower in the placebo group (**Table 3**). No adverse events were reported in either treatment group.

Effect of weekly CQ on haemoglobin change during follow-up

To assess the effect of weekly chloroquine on Hb recovery after clearance of peripheral parasitaemia, we compared change in Hb from Day 3 to Day 90 and Day 3 to Day 30 in the two arms. No significant differences in Hb change between the two groups were observed, either for the data pooled for both study years or for each study year separately (Day 90, CQ: +10.04 g/L [95% CI:6.66, 13.42] *vs.* placebo: +7.61 g/L [95% CI:2.88, 12.35]) (**Figure 2**). However, at Day 15, children receiving weekly CQ had an increase of 3.5 g/L (95% CI: 0.5, 6.4) compared to 0.8g/L (95% CI: -2.3, 3.9) in children in the placebo arm, but this difference did not reach statistical significance (p=0.2). The overall result was unchanged when adjusting for factors determined '*a priori*' (Hb at recruitment (Day 0), Hb at baseline (Day 3), age (in months), year of study, initial treatment received (CQ-SP *vs* ACT), and village group) using a random effects model. Furthermore, adding parasite density at recruitment, presence of sub-microscopic parasitaemia at any time point during follow-up, and presence of iron deficiency had no effect on the estimates of effect for CQ *vs*. placebo treatment during follow-up (data not shown).

Predictors of final haemoglobin at Day 90

In the absence of an effect of weekly CQ on Hb change after successful malaria treatment, we assessed what other factors might determine final Hb. Using simple linear regression significant positive associations were observed for parasite density at Day 0, better nutritional status, age, Hb at Day 0 at malaria diagnosis and Day 3 of successful treatment. Presence of iron deficiency at baseline was associated with significantly lower final Hb. There was no apparent effect of initial malaria treatment (CQ-SP *vs.* ACT) (**Table 4a**). In a final multiple regression model, Hb at Day 0 was the strongest predictor of final Hb and other independent predictors of final haemoglobin were presence of iron deficiency at Day 3, degree of stunting (height-for-age Z score), and age (in

months). Children with higher Hb at diagnosis and older children had greater final Hb. Children with iron deficiency at Day 3 had significantly lower final Hb with a mean difference of 12.1g/L (**Table 4b**).

Effect of initial anti-malaria treatment (CQ-SP *vs* ACT) on haemoglobin change

A secondary outcome of this study was Hb change from Day 3 to Day 30 and Day 90 in the placebo arm to determine whether initial anti-malaria treatment (CQ-SP or ACT) treatment affected macrophage iron loading in acute clinical malaria and its subsequent release. **Figure 3** depicts Hb change during follow up from Day 0 to Day 90 in the placebo arm by initial antimalarial treatment received. From Day 30 onwards there was a larger response in children who received anti-malaria therapy with CQ-SP compared to ACT. However, the drop in Hb from Day 0 to Day 3 and Day 7 in children who were initially treated with ACT was smaller than the drop in children initially treated with CQ-SP, although the difference was not significant resulting in similar levels of final Hb as indicated in the analysis to determine independent predictors of final Hb, in which Hb at Day 0 was the strongest predictor.

Sub-microscopic parasitaemia, inflammation, bone marrow response, haemoglobin change and treatment groups

Sub-microscopic parasitaemia was commonest in the first seven days of acute phase follow up (days 3-15). By Day 30, most were negative (77%). The prevalence of sub-microscopic parasitaemia at days 30 and 90 combined was 15.1% and there was no evidence of an effect of post-malaria treatment group or of initial anti-malarial treatment (**Figure 4a & 4b**). Most of the sub-microscopic parasitaemia at these time points was of a very low level (one to two parasites per microliter). However, higher parasite density at diagnosis was associated with an increased likelihood of subsequent sub-microscopic parasitaemia at day 30 or 90 with a one log increase in parasite count increasing the odds of having sub-microscopic parasitaemia by 1.6 (P=0.005). Although the presence/absence of sub-microscopic parasitaemia during follow up (days 30 & 90) was not associated with final Hb (**Table 4**), in children with high qPCR (quantitative PCR parasite count) parasite count (> 500/µL, N=3) at Day 30, Hb at Day 30 was significantly lower compared to

children with one to two parasites/µL or none at Day 30 (82.3g/L vs 102.8g/L; p = 0.01). Similarly, the mean Hb change was also reduced in those with persistent high parasitaemia 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). Bone marrow response as assessed by percentage of reticulocytes peaked at Day 15 (4.5% in both treatment groups combined), compared to similar levels of around 2% at all other measured time points (Days 3, 7, 15 & 90). There was no evidence of an effect of either post-malaria treatment group or of initial anti-malarial therapy (**Figure 5**) on bone marrow response (data not shown). Urinary neopterin, a product of activated macrophages and therefore a marker of a pro-inflammatory immune status, was strongly associated with qPCR parasite count at Day 30 (regression coefficient: 0.2 (95%CI: 0.08, 0.33), p = 0.003; adjusted R^2 = 0.4091) and was also associated with Hb at Day 30. There was no evidence of an effect of post-malaria treatment group or of initial anti-malarial treatment on neopterin concentrations (**Figure 6a & 6b**).

Discussion

Chloroquine is used in the treatment of several diseases in addition to its antimalarial use[28] [29]. However, we believe this to be the first study to assess its potential role in the management of post-malaria anaemia. We hypothesised that since malarial anaemia is essentially a systemic inflammatory response to infection[12], chloroquine, by suppressing plasmodium-induced inflammation, could enhance erythropoietic recovery. We tested this hypothesis by comparing two groups of children – one group receiving weekly chloroquine and the other group weekly placebo after complete clearance of peripheral parasitaemia as determined by microscopy. The primary outcome measure was the recovery in Hb at Days 30 and 90 compared to Day 3 Hb levels. Our results showed no significant difference in the Hb change between the two groups. The data was interrogated further by a random effects model taking into account random measurement errors and missing data. This confirmed that weekly chloroquine at 5mg/kg body weight did not improve Hb recovery after uncomplicated clinical malaria. There was a suggestion that weekly CQ may have increased the rate of recovery in Hb change at Day 15 accompanied by non-signficantly increased reticulocyte percentage.

A limitation of the study was the need to recruit participants over 2 malaria transmission seasons. In 2008 there was a reduced malaria transmission rate and thus fewer cases available for recruitment and also an apparent effect on the severity of malaria in terms of parasite densities at first detection and effect on haemoglobin concentrations, with a significantly higher baseline Hb in 2008. However, the change in design that was necessitated in 2008 did not change the primary outcome analysis .

The change in design in 2008 to all children receiving ACT anti-malarial treatment did not affect the analysis of the primary outcome. The change did, however, limit our ability to determine the effect of initial anti-malaria therapy on haemoglobin response in the placebo group. An effect of anti-malarial treatment CQ on Hb, when in the context of adequate parasite clearance as was observed, might have been expected to occur in the early period of recovery.

The recommended anti-inflammatory dose of CQ is 3-4mg/kg/day. However, due to lack of safety data for this condition and considering potential adverse effects, particularly retinal damage, we used the lower prophylactic dose of 5mg/kg/week. Using this low dose might have contributed to our inability to detect an effect of CQ. Drenou et al [30] reported the successful control of sideroblastic anaemia (a condition of reduced incorporation of iron into haem in the presence of sufficient or high iron) was for six years on 100mg daily chloroquine without need for blood transfusion.

We had hypothesized that chloroquine will impede macrophageal iron sequestration during the acute clinical episode and improve iron availability for the process of erythropoiesis. Urinary excretion of neopterin is markedly increased in individuals infected with malaria, and corresponds to concurrent activation of T cells and macrophages[31-33]. We measured the urinary neopterin at Days 3, 15 and 30 and expected to see a more rapid decline in neopterin level in the weekly chloroquine group than in the weekly placebo group; but no difference between the treatment groups was observed. It is possible that a difference may have occurred at an intermediate time point between Day 3 and Day 15, when levels had already almost normalised. Indeed, Brown et al [33] 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 our study, this would coincide with study Days 0 to 3. In future, it might be more informative to carry out more measurements during the first week of malaria diagnosis.

The participants were followed up for 90 days because reports had suggested that Hb continues to fall even after successful malaria treatment primarily due to continuing inflammation[34] [35]. In this study we found no evidence of persistent inflammation, except in the few cases with remaining parasitaemia at Day 30, who also had decreased absolute Hb and Hb change at Day 30 compared with children with no parasitaemia or parasite counts of 1-2/microlitre.

Our observation that children with higher Hb at diagnosis had greater Hb recovery is potentially paradoxical as one would have expected the reverse through statistical regression to the

mean and greater 'catch up' in children with lower starting Hb at Day 3. However, as the majority of iron used for Hb recovery is recycled[24], those with higher Hb at Day 0 have more iron available for recovery. Our non-provision of exogenous iron during the study may have restricted the ability of the more anaemic children to respond appropriately as suggested by our observation of reduced Hb recovery in children with apparent iron deficiency at Day 0. An earlier study in Gambian children showed improved haematological recovery in children treated for malaria and co-supplemented with iron compared to placebo[36]. However, we have also shown in Gambian children reduced iron absorption from supplementation in post-malarial anaemia, whilst also experiencing greater Hb recovery compared to supplemented children with iron-deficiency anaemia. Thus causing us to question the efficacy of iron supplementation given within 2 weeks of anti-malarial treatment[24]. Without the inclusion of a further randomised treatment arm of post-malarial treatment iron supplementation it is not possible to make further recommendations concerning the recent argument for the provision of iron supplementation to children in malaria endemic areas with regular malaria surveillance and treatment facilities[37].

Our finding that stunted children recovered Hb poorly is consistent with an earlier report of stunting being a determinant of the severity of malaria-associated anaemia[38]. In our population this effect was independent of iron status.

Age has been shown to be strongly associated with risk of severe anaemia in children with acute malaria; the risk decreasing with increasing age[39]. In addition, we have shown in this study that older children recover better from anaemia after an acute malaria episode than younger children. Paying special attention to younger children with malaria anaemia could reduce malaria related mortality in children.

In conclusion, weekly chloroquine administered for three months to children with mild malaria anaemia at a dose of 5mg/kg/week resulted in no improvement in erythropoietic recovery compared to placebo. Older children, children with higher initial Hb, less stunting at diagnosis, and without iron deficiency had better Hb recovery post-malaria. Similar to other reports in the

literature, urinary neopterin was elevated during acute malaria. The role of iron deficiency in erythropoietic recovery post malaria noted in this study suggests areas for further detailed investigation of the clinical value in administering both antimalarial and iron supplementation to children with malaria anaemia residing in areas of high iron deficiency and malaria burden.

Acknowledgements

The authors wish to acknowledge the contribution of all the members of the field team – Kabiru Ceesay, Edrisa Sinjanka, Lamin Ceesay, Lamin Sowe, Kalilu Sanneh, Bakary Sarr, Muhammed Sidibeh, Yahya Sanyang, the data staff especially Jahid Hassan and Musa Jarjou, the laboratory staff especially Musa Colley, Karamo Camara, Mustapha Bittaye, and Ebrimah Danso, all the drivers of the MRC Keneba, Ms Cheryl Eniyou O, Mrs Fatou Noho, Ms Jankey Jagne, all the village assistants, and the study subjects and their parents.

Authorship contributions

CVN, CPD and AMP conceived the study with input from SEC. CVN led all practical aspects of the work with logistic and funding support from SEM and AMP. CVN, SEC and AJF were responsible for the statistical analysis and interpretation. CVN and SEC drafted the paper with input and approval from all authors.

Disclosure of Conflicts of Interest

The authors have no conflict of interest.

Data sharing

The original dataset is available on request to the corresponding author and permission is dependent on the nature and purpose of intended use.

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TABLES

Table 1: Laboratory investigations conducted

Investigations	Specimen	Days collected[†]	Purpose
Microscopy for malaria parasite	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess parasitaemia
Erythrocyte zinc protoporphyrin	Blood	Days 0 & 3	To assess iron status
Full blood count	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess Hb change over time
Automated reticulocyte percentage	Blood	Days 3, 7, 15, 30, 45, 70, 90	To monitor bone marrow response
Quantitative PCR for *P.falciparum* parasites	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess sub-microscopic parasitaemia
Urinary neopterin concentration	Urine	Days 3, 15, 30	To assess macrophage activation as measure of immune mediated inflammation

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

Table 2: Baseline characteristics of all participants under surveillance

	2007	2008	Combined
	N=1445	N=1220	N=2665
Mean Age in months (SD)	42.2 (17.2)	40.7 (17.7)	41.5 (17.5)
Mean Weight-for-Height Z-scores (SD)	-1.06 (0.84)	-1.02 (0.85)	-1.04 (0.84)
Mean Weight-for-Age Z-scores (SD)	-1.48 (0.97)	-1.50 (0.94)	-1.49 (0.95)
Mean Height-for-Age Z-scores (SD)	-1.06 (1.21)	-1.17 (1.13)	-1.11 (1.17)
Mean Body Mass Index (kg/m^2) (SD)	14.6 (1.3)	14.7 (1.4)	14.6 (1.4)
Prevalence of a malaria rapid test positive result during surveillance*	105 (7.3)	49 (4.0)	154 (5.8)

*****only the first positive result is counted.*

Table 3: Baseline characteristics of children randomised to weekly chloroquine and weekly placebo in 2007 and 2008, pooled

Parameter	Chloroquine group N=50	Placebo group N=46
Male sex (%)	28 (56.0)	24 (52.2)
Age in months, mean (SD)	41.9 (18.1)	38.7 (17.8)
Height-for-Age Z-scores, mean (SD)	-1.19 (0.91)	-0.94 (1.20)
Weight-for-Age Z-scores, mean (SD)	-1.44 (0.95)	-1.35 (0.87)
Weight-for-Height z scores, mean (SD)	-0.94 (0.90)	-0.96 (0.79)
BMI (kg/m^2), mean (SD)	14.7 (1.4)	14.7 (1.3)
Parasite density at recruitment (day 0), geometric mean, (95% CI)	47,783 (31,617 - 71120)	32,496 (21,910, - 47,507)
Hb [g/L] at recruitment (Day 0), mean (SD)	103.9 (14.2)	101.5 (14.8)
Hb [g/L] at baseline (Day 3), mean (SD)	93.4 (9.6)	93.7 (10.2)
Iron deficient[1] at baseline (Day 3) (%)	23 (46)	23 (50)

1 *Iron deficiency was defined as Znpp ≥ 61µmol/mol of Hb &, MCV≤ 73fl & MCH ≤25pg*

Table 4a: Predictors of final haemoglobin at day 90 using univariable linear regression

Parameter	Regression coefficient (95%CI)	P-value
Parasite count at recruitment (day 0)	3.5 (1.1, 5.8)	0.004
Sex	-0.4 (-3.3, 2.5)	0.8
Iron deficiency at baseline (day 3)	-12.1 (-14.7, -9.5)	<0.001
Height-for-Age Z-scores (HAZ) <-2	3.2 (1.8, 4.6)	<0.001
Weight-for-Age Z-scores (WAZ) <-2	2.6 (1.0, 4.2)	0.001
Weight-for-Height (WHZ) z scores <-2	0.1 (-1.6, 1.8)	0.9
Age in months	0.4 (0.3, 0.4)	<0.001
Initial anti-malaria treatment arm	1.2 (-2.1, 4.4)	0.5
CQ *vs.* placebo randomisation group	-0.5 (-3.4, 2.5)	0.8
Hb [g/L] at recruitment (day 0)	0.6 (0.5, 0.6)	<0.001
Hb [g/L] at baseline (day 3)	0.7 (0.6, 0.8)	<0.001
Village	1.9 (0.9, 3.0)	<0.001
Presence of sub-microscopic parasitaemia at any time point during follow-up	0.6 (-7.1, 8.2)	0.9
Year of study	-1.3 (-4.4, 1.8)	0.4

Table 4b: Independent predictors of final haemoglobin (day 90) using multivariable linear regression

Parameter	Regression coefficient (95% CI)	P value
Day 0 Hb	0.44 (0.36-0.52)	<0.001
Presence of iron deficiency	-3.0 (-5.57, -0.43)	0.022
Height-for-age Z score <-2	1.48 (0.41, 2.56)	0.007
Age in months	0.21 (0.14, 0.28)	<0.001

Model: Adjusted r^2=0.46, F=76.6, Prob>F <0.001

Figure Legends

Figure 1: CONSORT Flow Diagram describing participants' movements in 2007 & 2008

Figure 2 Mean change in haemoglobin concentration at day 30 and day 90 from baseline at day 3 by treatment group for each year of study and for both years combined.

Error bars represent the 95% confidence intervals

Figure 3 Mean change in hemoglobin in the placebo arm by initial anti-malarial treatment group in 2007 study.

Change in Hemoglobin at day 3 is compared to haemoglobin concentration at presentation with malaria and initiation of anti-malaria treatment. Thereafter the comparison is with baseline levels at day 3 after completion of anti-malarial treatment. Error bars represent the 95% confidence intervals

Figure 4a. Percentage of participants with sub-microscopic malaria (qPCR) over time by post-malaria treatment group

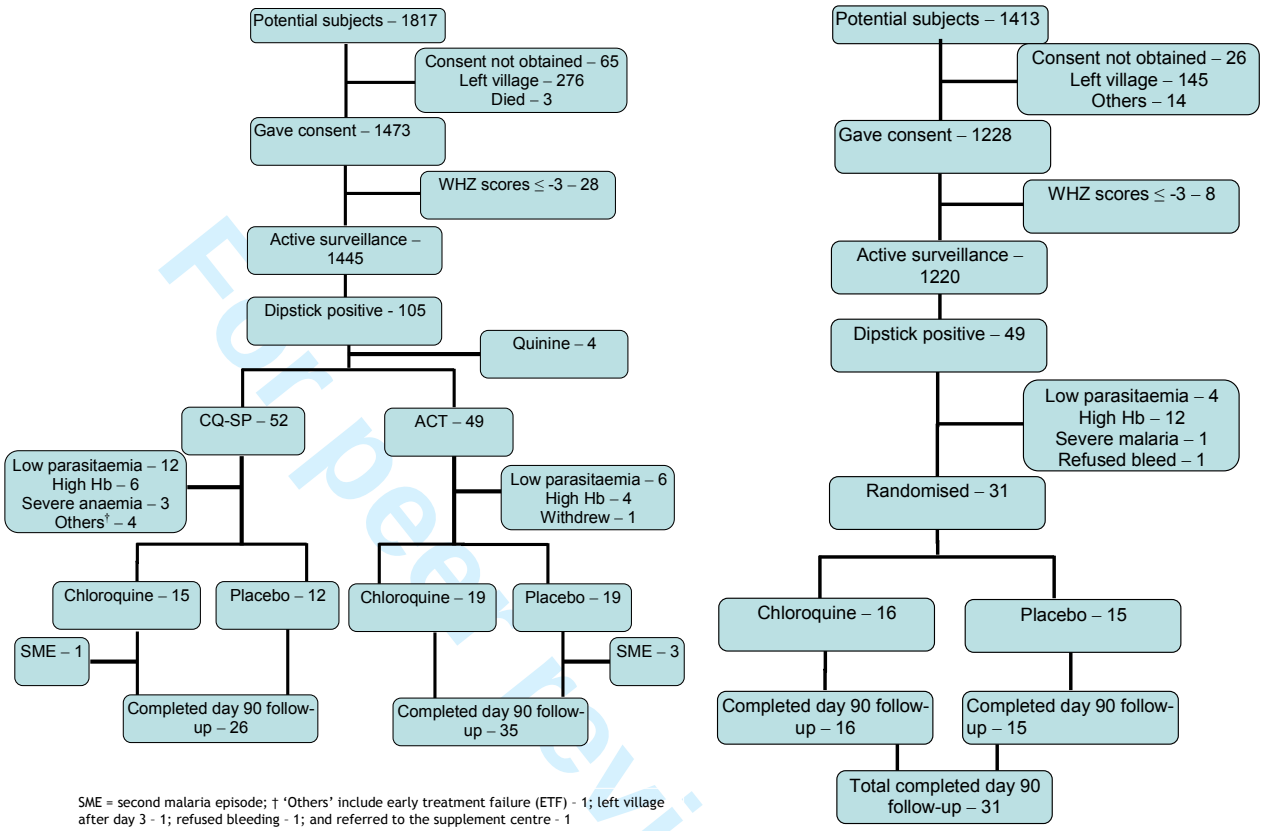
Figure 4b. Percentage of participants with sub-microscopic malaria (qPCR) over time in the placebo group (2007 study) by initial anti-malaria treatment

Figure 5. Reticulocyte percentage as an indicator of bone marrow response by treatment group for both years combined.

Figure 6a. Urinary neopterin by post-malaria treatment group

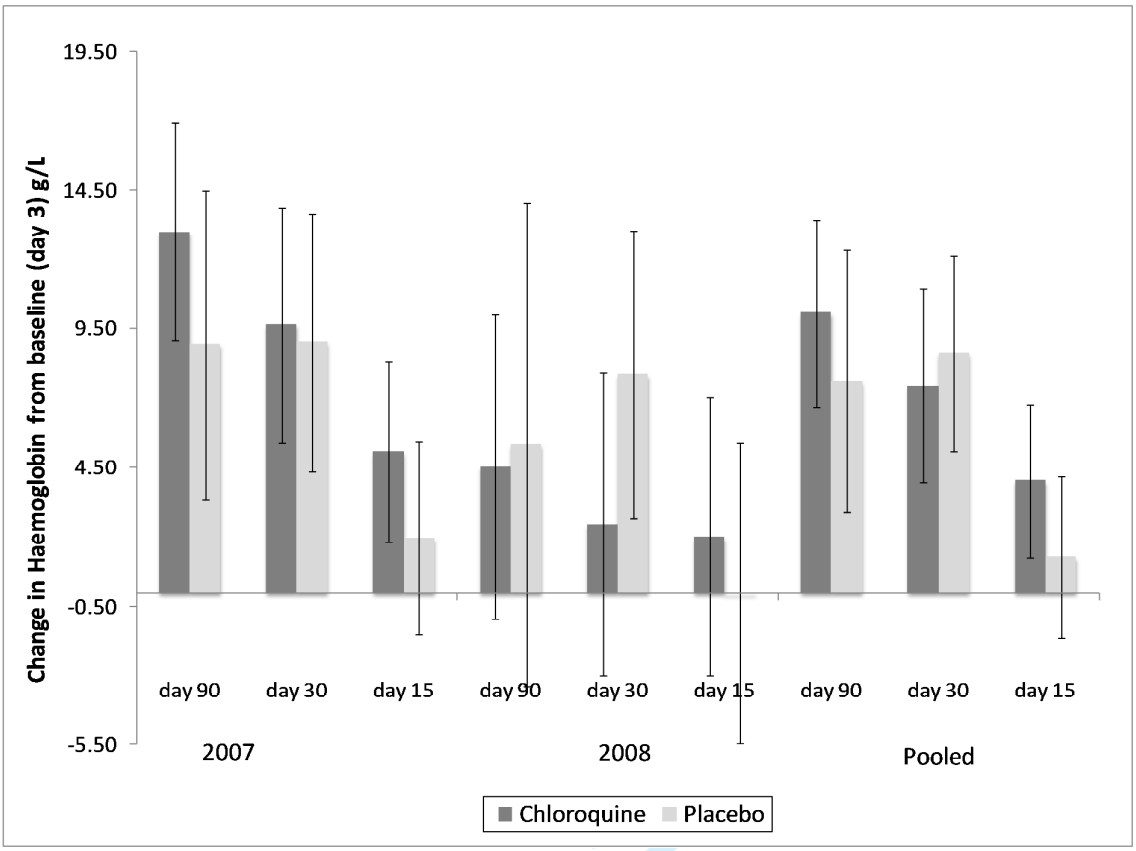
Figure 6b. Urinary neopterin in the placebo group (2007 study) by initial anti-malaria treatment

Figure 1



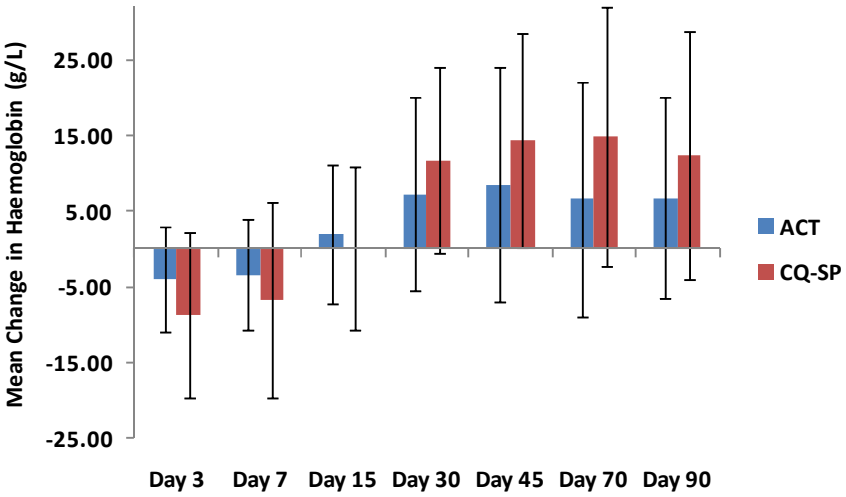
SME = second malaria episode; † 'Others' include early treatment failure (ETF) - 1; left village after day 3 - 1; refused bleeding - 1; and referred to the supplement centre - 1

Figure 2 Mean change in haemoglobin concentration at day 30 and day 90 from baseline at day 3 by treatment group for each year of study and for both years combined.



Error bars represent the 95% confidence intervals

Figure 3 Mean change in hemoglobin in the placebo arm by initial anti-malarial treatment group in 2007 study.



Change in Hemoglobin at day 3 is compared to haemoglobin concentration at presentation with malaria and initiation of anti-malaria treatment. Thereafter the comparison is with baseline levels at day 3 after completion of anti-malarial treatment. Error bars represent the 95% confidence intervals

Figure 4a. Percentage of participants with sub-microscopic malaria (qPCR) over time by post-malaria treatment group

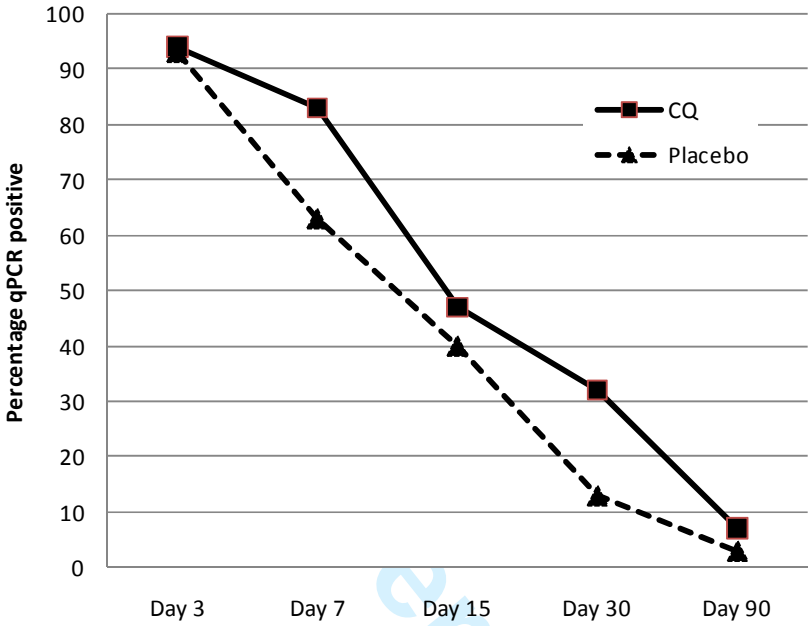


Figure 4b. Percentage of participants with sub-microscopic malaria (qPCR) over time in the placebo group (2007 study) by initial anti-malaria treatment

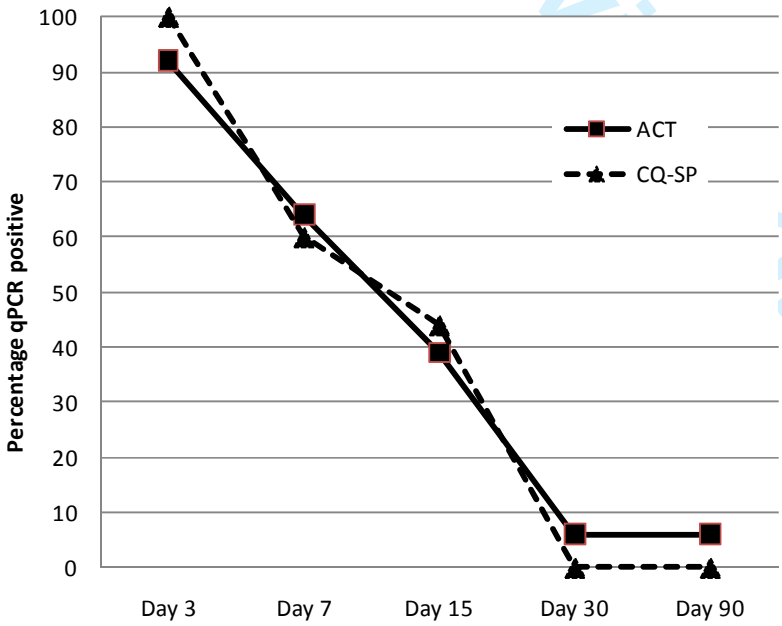


Figure 5. Reticulocyte percentage as an indicator of bone marrow response by treatment group for both years combined.

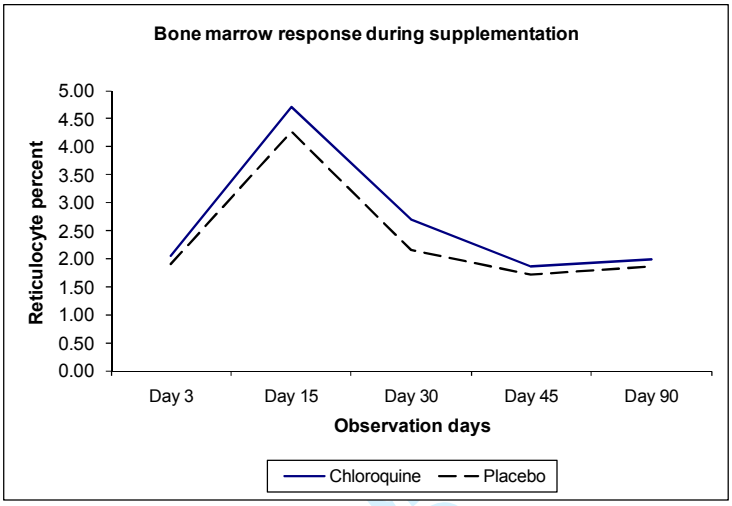


Figure 6a. Urinary neopterin by post-malaria treatment group

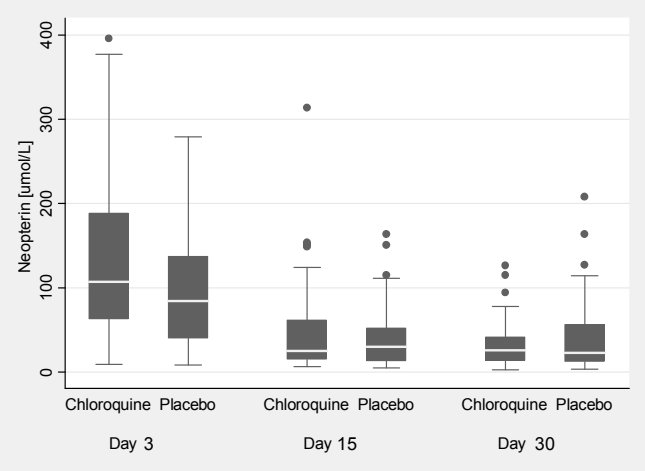
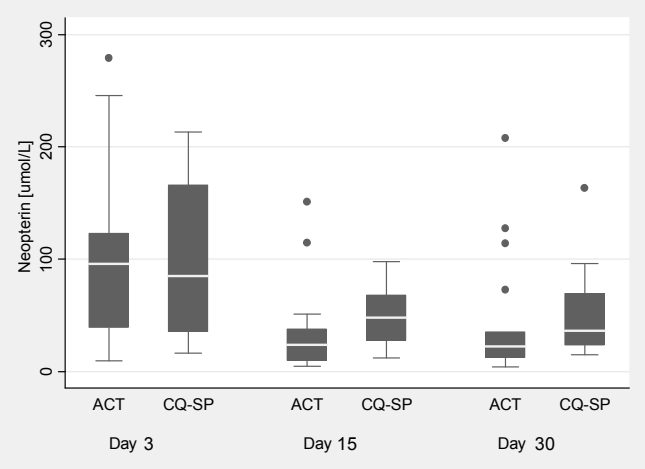


Figure 6b. Urinary neopterin in the placebo group (2007 study) by initial anti-malaria treatment



CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2-3
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	6-7
	2b	Specific objectives or hypotheses	7
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	8
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	8
Participants	4a	Eligibility criteria for participants	8-9
	4b	Settings and locations where the data were collected	8
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	**10-11** & 9-10
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	**11**, 9-10, & 12
	6b	Any changes to trial outcomes after the trial commenced, with reasons	N/A
Sample size	7a	How sample size was determined	11-12
	7b	When applicable, explanation of any interim analyses and stopping guidelines	N/A
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	10
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	10
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	10
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	10
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	10

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	10-11
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	12-13
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	12-13
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	14 & Fig1
	13b	For each group, losses and exclusions after randomisation, together with reasons	14
Recruitment	14a	Dates defining the periods of recruitment and follow-up	8 & 4
	14b	Why the trial ended or was stopped	N/A
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	14-15 &Table 3
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	15-18
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	15-18 Tables 3,4a, 4b, Figs 2-6
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	N/A
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	N/A
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	N/A
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	2, 19-21
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	19-21
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	19-21
Other information			
Registration	23	Registration number and name of trial registry	1
Protocol	24	Where the full trial protocol can be accessed, if available	N/A
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	1

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

BMJ open

Randomised controlled trial of weekly chloroquine to re-establish normal erythron iron flux and haemoglobin recovery in post-malarial anaemia

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2013-002666.R1
Article Type:	Research
Date Submitted by the Author:	11-Apr-2013
Complete List of Authors:	Cox, Sharon; London School of Hygiene & Tropical Medicine, MRC International Nutrition Group; Muhimbili Wellcome Programme, Nweneka, Chidi; MRC Keneba, Doherty, Conor; Royal Hospital for Sick Children, Fulford, Anthony; London School of Hygiene andTropical Medicine, MRC International Nutrition Group Moore, Sophie; London School of Hygiene andTropical Medicine, MRC International Nutrition Group Prentice, Andrew; London School of Hygiene andTropical Medicine, MRC International Nutrition Group; MRC Keneba,
Primary Subject Heading:	Haematology (incl blood transfusion)
Secondary Subject Heading:	Epidemiology, Nutrition and metabolism, Infectious diseases, Pharmacology and therapeutics
Keywords:	TROPICAL MEDICINE, Clinical trials < THERAPEUTICS, Anaemia < HAEMATOLOGY

Randomised controlled trial of weekly chloroquine to re-establish normal erythron iron flux and haemoglobin recovery in post-malarial anaemia

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Sources of support: This study was supported by the UK Medical Research Council (MC-A760-5QX00). The funder had no role in the design, analysis or reporting of this trial.

Running head: Chloroquine in post-malarial anaemia

Abbreviations used: ACT, co-artemether; CQ-SP, chloroquine plus sulphadoxine-pyrimethamine; Hb, haemoglobin; MCH, mean cell haemoglobin; MCV, mean cell volume; RCT, randomised controlled trial; SMA, severe malarial anaemia; ZnPP, zinc protoporphyrin.

Trial registration: The clinical trial registration number is NCT00473837 (ClinicalTrials.gov).

Abstract

Objective: To determine if low-dose weekly chloroquine (CQ) therapy improves recovery from malaria-associated anaemia.

Design: Proof of concept randomised clinical trial.

Setting: West Kiang District, Lower River Region, The Gambia

Participants: Children resident in participating communities, aged 12-72 months with uncomplicated malaria identified using active case detection over two consecutive malaria transmission seasons.

Interventions: In 2007 eligible children were randomised to chloroquine-sulphadoxine/pyrimethamine (CQ-SP) or co-artemether (ACT) anti-malarial therapy, and after parasite clearance on Day 3 subsequently re-randomised (double-blind) to weekly low-dose CQ (5mg/kg) or placebo. In 2008 all eligible children were treated with ACT and subsequently randomised to CQ or placebo.

Outcome measures: The primary outcome was change in haemoglobin from baseline (Day 3 of anti-malarial treatment) to Day 90 in the CQ and placebo treatment arms. Secondary outcomes were changes in urinary neopterin as a marker of macrophage activation, markers of erythropoietic response and prevalence of sub-microscopic parasitaemia. Change in haemoglobin in the placebo arm by initial anti-malarial treatment was also assessed.

Results: In 2007 101 children with uncomplicated malaria were randomised to anti-malarial treatment with CQ-SP or ACT and 65 subsequently randomised to weekly CQ or placebo. In 2008 all children received ACT anti-malarial treatment and 31 subsequently randomised to weekly CQ or placebo. Follow up to Day 90 was 96%. There was no effect of weekly CQ *vs.* placebo on change in haemoglobin at Day 90, (CQ: +10.04 g/L [95% CI:6.66/13.42] *vs.* placebo: +7.61 g/L [95% CI:2.88/12.35]). There was no effect on the secondary outcomes assessed, or effect of initial anti-malarial therapy on haemoglobin recovery. Higher Day 90 haemoglobin independently correlated with older age, not being stunted, higher haemoglobin at Day 0 and adequate iron status at Day 3.

Conclusions: Weekly low-dose CQ after effective anti-malarial treatment is not effective in improving recovery from post-malaria anaemia.

Article Focus

- To determine if 3 months of low-dose weekly chloroquine (CQ) (5mg/kg) therapy after effective treatment of uncomplicated malaria can improve haemoglobin recovery after malaria-associated anaemia in rural African children.
- To test the hypothesis that chloroquine may enhance haematologic recovery after a malaria episode through its anti-inflammatory and immuno-modulatory actions, anti-macrophageal iron loading effect, and anti-malarial effect (despite widespread resistance).

Key Messages

- Weekly low-dose CQ after effective anti-malarial treatment was not effective in improving recovery from mild to moderate post-malaria anaemia.
- Weekly low dose CQ at 5mg/kg had no clear effects on erythropoietic responses as determined by reticulocyte percentage, or immune-mediated inflammation as assessed by urinary neopterin, a marker of macrophage activation.

Strengths and limitations

- The strengths of this study are its randomised design and double blinding of the allocation to weekly CQ or placebo and that few subjects were lost to follow-up. The continuous follow-up and active case detection for all children ensured that malaria infections were detected early and within similar time-frames, plus resurgent or new clinical infections were excluded from the analysis of haemoglobin change. A strict malaria case definition was adhered to and the measurement of sub-microscopic parasitaemia over time allowed the effects of incomplete treatment to be modelled.
- A limitation of the study was the need to recruit participants over 2 malaria transmission seasons and the significantly reduced malaria transmission rate in 2008 and thus reduced recruitment which also appeared to affect the severity of malaria in terms of parasite

densities at first detection and haemoglobin concentrations. However, the confidence intervals of the effect size in the final multivariable analysis do not include the clinically significant effect size included in the sample size calculation and thus our observation is unlikely to be due to a type II error as a result of lack of power to detect a true effect.

- It is possible that the relatively low dose of CQ we used was insufficient to mediate the effects that we were testing for and we were unable to conduct pilot studies of CQ drug dosage and duration to determine the potential best treatment regime to test or of the timings of evaluation of possible mechanisms of effect.
- The change in design in 2008 to all children receiving ACT anti-malarial treatment did not affect the analysis of the primary outcome. The change did, however, limit our ability to determine the effect of initial anti-malaria therapy on haemoglobin response in the placebo group. An effect of anti-malarial treatment CQ on Hb, when in the context of adequate parasite clearance as was observed, might have been expected to occur in the early period of recovery.

Introduction

The precise contribution of malaria to anaemia is difficult to calculate mainly because many anaemia-causing conditions co-exist in children living in malaria-endemic countries. [1] Murphy and Breman [2] estimate the annual incidence of severe malaria anaemia (SMA) to be 1.42 to 5.66 million resulting in between 194,000 to 974,000 deaths in children younger than five years. SMA increases the risk of death in children with malaria [3], many of whom die shortly after admission before blood transfusion can be commenced [4 5] and after discharge in the community [6]. In addition, blood transfusion, the standard treatment for severe malaria anaemia exposes the child to the risk of transfusion-related infections like Human Immunodeficiency Virus (HIV) and hepatitis [7]. Among a nationally representative sample of children aged one to five years in The Gambia in 1999, 76% had a haemoglobin (Hb) less than 11g/dl and 15% had Hb less than 7g/dl [8]. It is likely that most of the cases of moderate to severe anaemia were due in great part to malaria [9-11]. A better understanding of the pathogenesis of malaria anaemia is required to improve its prevention and management.

Multiple immune and non-immune-mediated mechanisms cause anaemia during and after a malaria episode[12] including the destruction of non-parasitized erythrocytes which may account for more than 90% of erythrocyte loss[13 14]. One important component is inflammation-induced iron delocalisation which is characterised by sequestration of iron by the monocyte-macrophage system and hepatocytes, resulting in a state of pseudo-iron deficiency; a state that reverses after the inflammation has subsided[12]. The trans-membrane protein ferroportin is the exclusive route for cellular iron efflux and is responsible for releasing absorbed iron from enterocytes and sequestered iron from macrophages[12 15 16]. Hepcidin, the master-regulator of iron metabolism which responds both to iron status and inflammation[15], blocks ferroportin. Clinically, malaria-induced inflammation has been shown to be associated with a hepcidin-mediated reduction in intestinal absorption of iron. [17] Inflammatory cytokines, particularly IL-6 and IL-1 stimulate hepcidin release[18 19] resulting both in reduced iron absorption of dietary iron and impaired macrophageal release of iron; the latter effect being quantitatively much more significant as macrophageal iron recycling and transport to the

erythron is 25-30 fold greater than the influx of dietary iron. In this way hepcidin is the mediator of the anaemia of chronic disease[15]. TNF-α could also play a role in ferroportin-mediated iron transfer independent of hepcidin[20]. Rapid resolution of malaria-induced inflammation could therefore limit the degree and duration of malaria anaemia by reducing the duration of iron delocalisation.

Chloroquine is a cheap and safe antimalarial with proven anti-inflammatory properties; and has been shown to reduce iron delocalisation *in vitro*[21 22]. We hypothesised that chloroquine may enhance haematologic recovery after a malaria episode through its anti-inflammatory and immuno-modulatory actions, anti-macrophageal iron loading effect, and anti-malarial effect (despite widespread resistance). We designed a proof of concept study to investigate the potential use of chloroquine in the management of mild post malaria anaemia.

Materials and Methods

Study Design and population

This study was a population-based randomised, controlled trial (RCT) in children aged 12 to 72 months conducted over two malaria seasons in 2007 and 2008 in West Kiang District, Lower River Region, The Gambia. The study was initially designed and conducted as a 2x2 randomised placebo-controlled trial in 2007 but had to be changed, due to changes in national malaria treatment guidelines, to a two-arm RCT during the 2nd malaria transmission season in 2008. In the 2x2 phase of the study, children with malaria were initially openly randomised to receive a therapeutic dose regime of chloroquine plus sulphadoxine-pyrimethamine (CQ-SP) or co-artemether (ACT) (Day 0). After treatment, on Day 3, children were subsequently randomised (double blind) to weekly chloroquine or placebo until Day 90 (**Figure 1**). In the two-arm phase, all enrolled children with malaria were treated with ACT and then randomised and followed up as before (**Figure 1**). A combination of active and passive case detection was used to identify children in participating communities with malaria using a standard case definition of clinical malaria (see below).

The climate of this district is typical of sub-Sahelian Africa with a long dry season lasting from November to June and a relatively short rainy season lasting from July to October. Malaria transmission is seasonal, hyperendemic and follows the rainy season, occurring between July and December. The prevalence of children experiencing one or more malaria episodes among a cohort of 1002 children aged between 1-6 years under active malaria surveillance during a population-based study in 2003 was 34% (Doherty et al. Unpublished data). During the study period standard malaria control activities in the district consisted of insecticide treated bednet distribution and case finding and treatment. In 2008 the standard first-line antimalarial treatment regimen for uncomplicated malaria in The Gambia was changed from CQ-SP to co-artemether.

Inclusion & exclusion criteria

Eligibility for malaria surveillance and subsequent possible enrolment into the RCT included all children in the participating communities, aged between 12 and 72 months at the start of the

surveillance period and who were not severely wasted, defined as weight-for-height Z scores less than -3SD.

Inclusion criteria for enrolment included: 1) uncomplicated malaria at Day 0, defined as a history of fever 48 hours prior to presentation or a measured temperature of 37.5°C or more with peripheral parasitaemia (asexual forms of *P. falciparum* ≥500/µL by microscopy) on Day 0 of the study; and 2) for randomisation to the post-malaria intervention, children had to have a Hb between 69g/L - 110g/L on Day 3 with no peripheral parasitaemia.

Children were excluded from the RCT if they were: 1) unable to take oral medications; 2) had features of severe malaria;[1] 3) had a known haemoglobinopathy; 4) were enrolled in another project; 5) had already received antimalarial drugs from other sources outside the project team; or 6) were prescribed other drugs with potential antimalarial or anti-anaemic effects such as co-trimoxazole and haematinics.

Following randomisation to weekly CQ or placebo children were withdrawn from the study if: 1) they developed a second malaria episode or had life-threatening symptoms, more severe disease or serious adverse reactions; 2) left the study area for more than 14 days; 3) received antimalarials from other sources; or 4) Hb fell below 70g/L.

Field and clinical procedures

To effect the surveillance protocol, the study villages were divided into geographically adjacent clusters supervised by a resident study nurse who visited each village morning and evening and provided 24-hour clinical coverage to all enrolled children in their cluster. The active surveillance consisted of twice weekly temperature monitoring of enrolled children by village assistants; while the passive surveillance consisted of mothers referring sick children to the visiting study nurse. Children with fever – reported or measured – were screened for malaria using a rapid diagnostic test (MRDT) (Cortez diagnostics, INC, USA) after clinical evaluation. Children testing negative to the MRDT were treated based on the likely diagnosis from the symptoms and signs. Children testing positive to the MRDT were commenced on antimalarials immediately (either CQ-SP or ACT in 2007,

and ACT in 2008 for three days) and directly observed by the study nurse and managed as appropriate. Finger prick blood samples (days 7, 15, 30 & 70) were collected in an EDTA microtainer while venous blood (2mls – days 0 & 90; and 5mls – days 3 & 45) samples were collected in an EDTA vacutainer and sent to the study laboratory on the same day. Co-morbidities were treated as appropriate, avoiding co-trimoxazole and haematinics. For the subsequent visits after initial malaria diagnosis at Day 0, children were seen by the study nurse and blood samples and clinical data collected as per protocol. Only children who fulfilled the inclusion criteria for advancement to the weekly chloroquine/weekly placebo phase of the study were sampled after Day 3. These children were also followed up on days 15, 30, 45, 70 (except in 2008) and 90.

Randomisation and blinding

The randomisation to malaria treatment group in 2007 (CQ-SP or ACT) was open (non-blinded to trial staff, investigators or participants) and was done by sequential assignment as children were identified. The block randomisation to the post-malaria treatment of weekly CQ or placebo in both 2007 and 2008 was double blinded and was carried out in blocks of eight. The randomisation codes were generated by a staff member independent of the study team and held by the external trial monitor. Treatment codes were labelled A to H and placed in sequentially numbered, opaque sealed envelopes held by the study nurses. Allocation to the treatment was by matching the code in the envelope to a bottle of the intervention labelled with the same code and then labelled with the subject ID.

Interventions

For children openly randomised to malaria treatment at Day 0 with CQ-SP, children received choroquine syrup (Chlormal®, Medreich Sterilab Ltd, Avalahalli, Bangalore, India) consisting of 50mg chloroquine base per 5mls as chloroquine phosphate at a dose of 10mg/kg for 3 days (days 0-2). SP (Micro Laboratories, India) was co-administered as a 525 mg tablet (500mg sulfadoxine and 25mg pyrimethamine) on Day 0 only at a dose of ½ tablet/10kg. Children who were randomised to ACT treatment in 2007 and all children in 2008 received co-artemether (Coartem®) tablets (Beijing

Novartis Pharma Ltd, Beijing China for Novartis Pharma AG, Basle, Switzerland) as blister packets of 6 tablets per card with each tablet containing 20mg artemether and 120mg lumefantrine. The treatment dose comprised a stat dose on Day 0 and then 8 hours later, followed by twice daily doses for the next two days with doses depending on body weight (5 - <15 kg – one tablet/dose; 15 - <25kg – 2 tablets/dose; 25 - <35kg – 3 tablets per dose).

During the second blinded phase of the trial children received either weekly CQ syrup (as above) but at a dose of 5mg/kg or matching placebo syrup (prepared by Royal Victoria Teaching Hospital pharmaceutical laboratory in collaboration with Atlantic Pharmaceuticals Limited, Latrikunda Sabiji, The Gambia) The syrups were in similar amber coloured bottles with matching caps and labels. Doses were administered in the child's home under direct observation by the study nurses who waited for at least 10 minutes after administering the drugs to ensure that they were not vomited.

Primary and secondary outcomes

The primary outcome measure was Hb change from Day 3 post treatment of malaria episode to Day 90 in the weekly CQ and placebo arms.

The secondary outcomes were to compare between the weekly CQ and placebo arms: (1-i) changes in erythropoietic response between Day 3 and Day 30; and (1-ii) between day 3 and day 90; (2) changes in urinary neopterin from Day 3 to Day 30 as a marker of macrophage activation; (3) prevalence of sub-microscopic malaria parasitaemia using PCR at each time point, and; (4) Hb change from Day 3 to Day 90 in the two placebo arms to investigate the effects of initial malaria treatment therapy.

Sample size

During initial study design we used a working assumption of a standard deviation (SD) for change in Hb of 15 g/l[23]. With power 80% and significance level set at 5% we estimated the sample sizes required to detect varying effect sizes for the primary outcome. A sample size of 65 per arm

was estimated to be able to detect an effect size of 7.5g/l. This sample size was thought to be achievable and the effect size of likely public health significance.

Laboratory procedures:

Table 1 describes the different laboratory analysis conducted. Zinc protoporphyrin (ZnPP) as a marker of iron status was measured on fresh whole blood using a fluorometer (AVIV Biomedicals, Lakewood, NJ, USA). Full blood count (FBC), haemoglobin and automated reticulocyte count were performed using a Cell Dyne 3700 (Abbott Laboratories. Abbott Park, Illinois, USA). Iron deficiency was defined using 3 parameters; mean cell volume (MCV), mean cell haemoglobin (MCH) and ZnPP. The cut off values for the different parameters employed were: ≥61µmol/mol of Hb for ZnPP [24], ≤73fl for MCV [25] and ≤25pg for MCH [25]. Combining these parameters has been shown to increase their sensitivity and specificity to identify iron deficiency [26]. Malaria parasites were identified in a thick peripheral blood smear stained with Giemsa overnight and read by light microscope with x100 magnification in oil immersion. Parasite DNA extraction and subsequent quantitative PCR amplification were carried out using established protocols [27]. In brief, DNA extraction was done using the QIamp DNA Mini Kit (Qiagen™ Ltd. UK) according to the manufacturer's instructions with minor modifications. Extracted DNA samples were stored at -20°C. Quantification of parasite density was done with a TaqMan assay employing a fluorogenic probe to detect specific parasite products and using an Opticon® 2 Real Time PCR machine and Opticon™ version 3.1 software (Bio-Rad Laboratories, Inc., USA). Primers and probes were synthesized by Applied Biosystems, UK. Urinary neopterin levels were measured using an ELISA technique (BRAHMS Neopterin EIA, B.R.A.H.M.S, Germany). The stored urine was allowed to thaw at room temperature and then diluted 1:100 and the rest of the procedure was according to the manufacturer's instructions.

Statistical analysis:

Delta haemoglobin summary statistics are presented as means and standard deviations (SD) for normally distributed continuous variables, 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 are presented for log-transformed variables. Two-sided student's t tests were used to compare results between two treatment arms.

Visual inspection of Hb change between baseline at Day 3 and later time points by treatment arm suggested that by Day 30, Hb change in both treatment arms had reached a plateau. To fully determine the effect of treatment group on Hb change we used random effects models using the repeat measures at day 30 and day 90. Initial malaria treatment group (CQ-SP vs ACT), Hb at recruitment (Day 0 Hb), baseline Hb (Day 3), age in months, year of study, and the child's village were included in models "*a priori*" and hence adjusted for.

Linear regression was used to investigate further potential predictors of Hb change in the study population. First, 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 to assess confounding. The final regression model consisted of only the variables that maintained a significant association with Hb in the multiple regression models. All analyses were carried out using STATA version 8 (StataCorp, College Station, TX, USA).

Ethical clearance

The Scientific Coordinating Committee of the Medical Research Council (UK), The Gambia, and the MRC/Gambia Government Joint Ethical Committee approved the study (SCC 1076). Written informed consent was obtained from parents/guardians of the children prior to enrolment. The clinical trial registration number is NCT00473837 (ClinicalTrials.gov).

Results

Characteristics of the study population under malaria surveillance

Figure 1 describes the flow of participants and reasons for exclusions in the study. In 2007, 1445 of 1817 (80%) potentially eligible children identified through the Keneba MRC DSS database from 14 villages were included in the malaria surveillance. In 2008, 1220 of the 1413 (86%) identified children from 5 of the original villages were included in the malaria surveillance (smaller villages were excluded as they had contributed few cases in 2007). **Table 2** describes the characteristics of the children placed under surveillance for malaria. The proportion of children with a positive malaria rapid test during surveillance (only first event per child counted) fell from 7.3% (105/1445) in 2007 to 4% (49/1220) in 2008. In the first year of the study, 101 (96%) of the children with a positive malaria rapid test were randomised to CQ-SP or ACT treatment, and 65 (64%) subsequently randomised at Day 3 to weekly chloroquine or placebo, with all children completing follow up until Day 30 and 33/34 completing follow-up until Day 90 in the weekly CQ and 28/31 in the weekly placebo group. In the second year of the study 31 of the 49 (63%) cases with a first positive malaria rapid test were subsequently randomised to weekly CQ or placebo after treatment with ACT, all of whom completed follow up until Day 90 (16 CQ & 15 placebo).. Thus 96 children were included in the analysis of the primary and secondary outcomes, except for the analysis of effect of initial malaria treatment within the placebo arms which included 31 children.

Table 3 describes the characteristics of participants randomised to weekly chloroquine and weekly placebo for both years combined. Demographic and anthropometric characteristics were similar between the groups as was baseline Hb at Day 3. The prevalence of iron deficiency measured at baseline was 43.1% (28/65) in 2007 and 58.1% (18/31) in 2008 with a similar prevalence between the CQ/placebo groups within each year (data not shown) and in both years combined. However parasite density was lower in the placebo group (**Table 3**). No adverse events were reported in either treatment group.

Effect of weekly CQ on haemoglobin change during follow-up

To assess the effect of weekly chloroquine on Hb recovery after clearance of peripheral parasitaemia, we compared change in Hb from Day 3 to Day 90 and Day 3 to Day 30 in the two arms. No significant differences in Hb change between the two groups were observed, either for the data for both study years or for each study year separately (both years combined: change in Hb at Day 90, CQ: +10.04 g/L [95% CI:6.66, 13.42] *vs.* placebo: +7.61 g/L [95% CI:2.88, 12.35]) (**Figure 2**). Thus the effect size of the CQ treatment was 2.43 g/L [95& CI -3.38, 8.24] compared to the 7.5g/L we included in the sample size calculation. However, at Day 15, children receiving weekly CQ had an increase of 3.5 g/L (95% CI: 0.5, 6.4) compared to 0.8g/L (95% CI: -2.3, 3.9) in children in the placebo arm, but this difference did not reach statistical significance (p=0.2). The lack of effect of weekly CQ remained when adjusting for factors determined '*a priori*' (Hb at recruitment (Day 0), Hb at baseline (Day 3), age (in months), year of study, initial treatment received (CQ-SP *vs* ACT), and village group) using a random effects repeated measures model including Hb measured at day 30 and day 90 (coefficient =1.43 [95% CI: -1.70, 4.57). Furthermore, adding parasite density at recruitment, presence of sub-microscopic parasitaemia at any time point during follow-up, and presence of iron deficiency had no effect on the estimates of effect for CQ *vs*. placebo treatment during follow-up (data not shown).

Predictors of haemoglobin at Day 90

In the absence of an effect of weekly CQ on Hb change after successful malaria treatment, we assessed what other factors might determine final Hb. Using simple linear regression significant positive associations were observed for parasite density at Day 0, better nutritional status, age, Hb at Day 0 at malaria diagnosis and Day 3 of successful treatment. Presence of iron deficiency at baseline was associated with significantly lower final Hb. There was no apparent effect of initial malaria treatment (CQ-SP *vs.* ACT) (**Table 4a**). In a final multiple regression model, Hb at Day 0 was the strongest predictor of final Hb and other independent predictors of final haemoglobin were presence of iron deficiency at Day 3, degree of stunting (height-for-age Z score), and age (in months). Children with higher Hb at diagnosis and older children had greater final Hb. Children with iron deficiency at Day 3 had significantly lower final Hb with a mean difference of 12.1g/L (**Table 4b**).

Effect of initial anti-malaria treatment (CQ-SP *vs* ACT) on haemoglobin change

A secondary outcome of this study was Hb change from Day 3 to Day 30 and Day 90 in the placebo arm to determine whether initial anti-malaria treatment (CQ-SP or ACT) treatment affected macrophage iron loading in acute clinical malaria and its subsequent release. As indicated in the analysis of Hb at Day 90 there was no significant effect of inital anti-malarial treatment. Please see Supplementary **Figure 2** depicting Hb change during follow up from Day 0 to Day 90 in the placebo arm by initial antimalarial treatment received and the accompanying text.

Sub-microscopic parasitaemia, inflammation, bone marrow response, haemoglobin change and treatment groups

Sub-microscopic parasitaemia was commonest in the first seven days of acute phase follow up (days 3-15). By Day 30, most were negative (77%). The prevalence of sub-microscopic parasitaemia at days 30 and 90 combined was 15.1% and there was no evidence of an effect of post-malaria treatment group or of initial anti-malarial treatment (**Figure 3 & Supplementary Figure 2**). Most of the sub-microscopic parasitaemia at these time points was of a very low level (one to two parasites per microliter). However, higher parasite density at diagnosis was associated with an increased likelihood of subsequent sub-microscopic parasitaemia at day 30 or 90 with a one log increase in parasite count increasing the odds of having sub-microscopic parasitaemia by 1.6 (P=0.005). Although the presence/absence of sub-microscopic parasitaemia during follow up (days 30 & 90) was not associated with final Hb (**Table 4**), in children with high qPCR (quantitative PCR parasite count) parasite count (> 500/µL, N=3) at Day 30, Hb at Day 30 was significantly lower compared to children with one to two parasites/µL or none at Day 30 (82.3g/L vs 102.8g/L; p = 0.01). Similarly, the mean Hb change was also reduced in those with persistent high parasitaemia 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). Bone marrow response as assessed by percentage of reticulocytes peaked at Day 15 (4.5% in both treatment groups combined), compared to similar levels of around 2% at all other measured time points (Days 3, 7, 15 & 90). There was no evidence of an effect of either post-malaria treatment group (**Figure 4**)

or of initial anti-malarial therapy (data not shown)on bone marrow response. Urinary neopterin, a product of activated macrophages and therefore a marker of a pro-inflammatory immune status, was strongly associated with qPCR parasite count at Day 30 (regression coefficient: 0.2 (95%CI: 0.08, 0.33), p = 0.003; adjusted R^2 = 0.4091) and was also associated with Hb at Day 30. There was no evidence of an effect of post-malaria treatment group (**Figure 5)** or of initial anti-malarial treatment on neopterin concentrations (**Figure S3**).

Discussion

Chloroquine is used in the treatment of several diseases in addition to its antimalarial use[28] [29]. However, we believe this to be the first study to assess its potential role in the management of post-malaria anaemia. We hypothesised that since malarial anaemia is essentially a systemic inflammatory response to infection[12], chloroquine, by suppressing plasmodium-induced inflammation, could enhance erythropoietic recovery. We tested this hypothesis by comparing two groups of children – one group receiving weekly chloroquine and the other group weekly placebo after complete clearance of peripheral parasitaemia as determined by microscopy. The primary outcome measure was the recovery in Hb at Days 30 and 90 compared to Day 3 Hb levels. Our results showed no significant difference in the Hb change between the two groups. The data was interrogated further by a random effects model modelling data at Day 30 and Day 90 simultaneously whilst adjusting for Hb at Day 3 and taking into account random measurement errors and missing data. This confirmed that weekly chloroquine at 5mg/kg body weight did not improve Hb recovery after uncomplicated clinical malaria. There was a suggestion that weekly CQ may have increased the rate of recovery in Hb change at Day 15 accompanied by non-signficantly increased reticulocyte percentage.

A limitation of the study was the need to recruit participants over 2 malaria transmission seasons. In 2008 there was a reduced malaria transmission rate and thus fewer cases available for recruitment and also an apparent effect on the severity of malaria in terms of parasite densities at first detection and effect on haemoglobin concentrations, with a significantly higher baseline Hb in 2008. However, the change in design that was necessitated in 2008 did not change the primary outcome analysis. Furthermore, the confidence intervals of the observed effect size in the final multivariable analysis do not include the effect size included in the sample size calculation and thus our observation is unlikely to be due to a type II error as a result of lack of power to detect a true effect of this magnitude.

The change in design in 2008 to all children receiving ACT anti-malarial treatment did not affect the analysis of the primary outcome. The change did, however, limit our ability to determine the effect of initial anti-malaria therapy on haemoglobin response in the placebo group. An effect of anti-malarial treatment CQ on Hb, when in the context of adequate parasite clearance as was observed, might have been expected to occur in the early period of recovery.

The recommended anti-inflammatory dose of CQ is 3-4mg/kg/day. However, due to lack of safety data for this condition and considering potential adverse effects, particularly retinal damage, we used the lower prophylactic dose of 5mg/kg/week. Using this low dose might have contributed to our inability to detect an effect of CQ. Drenou et al [30] reported the successful control of sideroblastic anaemia (a condition of reduced incorporation of iron into haem in the presence of sufficient or high iron) was for six years on 100mg daily chloroquine without need for blood transfusion.

We had hypothesized that chloroquine will impede macrophageal iron sequestration during the acute clinical episode and improve iron availability for the process of erythropoiesis. Urinary excretion of neopterin is markedly increased in individuals infected with malaria, and corresponds to concurrent activation of T cells and macrophages[31-33]. We measured the urinary neopterin at Days 3, 15 and 30 and expected to see a more rapid decline in neopterin level in the weekly chloroquine group than in the weekly placebo group; but no difference between the treatment groups was observed. It is possible that a difference may have occurred at an intermediate time point between Day 3 and Day 15, when levels had already almost normalised. Indeed, Brown et al [33] 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 our study, this would coincide with study Days 0 to 3. In future, it might be more informative to carry out more measurements during the first week of malaria diagnosis.

The participants were followed up for 90 days because reports had suggested that Hb continues to fall even after successful malaria treatment primarily due to continuing inflammation[34]

[35]. In this study we found no evidence of persistent inflammation, except in the few cases with remaining parasitaemia at Day 30, who also had decreased absolute Hb and Hb change at Day 30 compared with children with no parasitaemia or parasite counts of 1-2/microlitre.

Our observation that children with higher Hb at diagnosis had greater Hb recovery is potentially paradoxical as one would have expected the reverse through statistical regression to the mean and greater 'catch up' in children with lower starting Hb at Day 3. However, as the majority of iron used for Hb recovery is recycled[24], those with higher Hb at Day 0 have more iron available for recovery. Our non-provision of exogenous iron during the study may have restricted the ability of the more anaemic children to respond appropriately as suggested by our observation of reduced Hb recovery in children with apparent iron deficiency at Day 0. An earlier study in Gambian children showed improved haematological recovery in children treated for malaria and co-supplemented with iron compared to placebo[36]. However, we have also shown in Gambian children reduced iron absorption from supplementation in post-malarial anaemia, whilst also experiencing greater Hb recovery compared to supplemented children with iron-deficiency anaemia. Thus causing us to question the efficacy of iron supplementation given within 2 weeks of anti-malarial treatment[24]. Without the inclusion of a further randomised treatment arm of post-malarial treatment iron supplementation it is not possible to make further recommendations concerning the recent argument for the provision of iron supplementation to children in malaria endemic areas with regular malaria surveillance and treatment facilities[37].

Our finding that stunted children recovered Hb poorly is consistent with an earlier report of stunting being a determinant of the severity of malaria-associated anaemia[38]. In our population this effect was independent of iron status.

Age has been shown to be strongly associated with risk of severe anaemia in children with acute malaria; the risk decreasing with increasing age[39]. In addition, we have shown in this study that older children recover better from anaemia after an acute malaria episode than younger children.

Paying special attention to younger children with malaria anaemia could reduce malaria related mortality in children.

In conclusion, weekly chloroquine administered for three months to children with mild malaria anaemia at a dose of 5mg/kg/week resulted in no improvement in erythropoietic recovery compared to placebo. Older children, children with higher initial Hb, less stunting at diagnosis, and without iron deficiency had better Hb recovery post-malaria. Similar to other reports in the literature, urinary neopterin was elevated during acute malaria. The role of iron deficiency in erythropoietic recovery post malaria noted in this study suggests areas for further detailed investigation of the clinical value in administering both antimalarial and iron supplementation to children with malaria anaemia residing in areas of high iron deficiency and malaria burden.

Acknowledgements

The authors wish to acknowledge the contribution of all the members of the field team – Kabiru Ceesay, Edrisa Sinjanka, Lamin Ceesay, Lamin Sowe, Kalilu Sanneh, Bakary Sarr, Muhammed Sidibeh, Yahya Sanyang, the data staff especially Jahid Hassan and Musa Jarjou, the laboratory staff especially Musa Colley, Karamo Camara, Mustapha Bittaye, and Ebrimah Danso, all the drivers of the MRC Keneba, Ms Cheryl Eniyou O, Mrs Fatou Noho, Ms Jankey Jagne, all the village assistants, and the study subjects and their parents.

Authorship contributions

CVN, CPD and AMP conceived the study with input from SEC. CVN led all practical aspects of the work with logistic and funding support from SEM and AMP. CVN, SEC and AJF were responsible for the statistical analysis and interpretation. CVN and SEC drafted the paper with input and approval from all authors.

Disclosure of Conflicts of Interest

The authors have no conflict of interest.

Data sharing

The original dataset is available on request to the corresponding author and permission is dependent on the nature and purpose of intended use.

Funding

UK Medical Research Council

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TABLES

Table 1: Laboratory investigations conducted

Investigations	Specimen	Days collected[†]	Purpose
Microscopy for malaria parasite	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess parasitaemia
Erythrocyte zinc protoporphyrin	Blood	Days 0 & 3	To assess iron status
Full blood count	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess Hb change over time
Automated reticulocyte percentage	Blood	Days 3, 7, 15, 30, 45, 70, 90	To monitor bone marrow response
Quantitative PCR for *P.falciparum* parasites	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess sub-microscopic parasitaemia
Urinary neopterin concentration	Urine	Days 3, 15, 30	To assess macrophage activation as measure of immune mediated inflammation

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

Table 2: Baseline characteristics of all participants under surveillance

	2007	2008	Combined
	N=1445	N=1220	N=2665
Mean Age in months (SD)	42.2 (17.2)	40.7 (17.7)	41.5 (17.5)
Mean Weight-for-Height Z-scores (SD)	-1.06 (0.84)	-1.02 (0.85)	-1.04 (0.84)
Mean Weight-for-Age Z-scores (SD)	-1.48 (0.97)	-1.50 (0.94)	-1.49 (0.95)
Mean Height-for-Age Z-scores (SD)	-1.06 (1.21)	-1.17 (1.13)	-1.11 (1.17)
Mean Body Mass Index (kg/m^2) (SD)	14.6 (1.3)	14.7 (1.4)	14.6 (1.4)
Prevalence of a malaria rapid test positive result during surveillance*	105 (7.3)	49 (4.0)	154 (5.8)

*****only the first positive result is counted.*

Table 3: Baseline characteristics of children randomised to weekly chloroquine and weekly placebo in 2007 and 2008, pooled

Parameter	Chloroquine group N=50	Placebo group N=46
Male sex (%)	28 (56.0)	24 (52.2)
Age in months, mean (SD)	41.9 (18.1)	38.7 (17.8)
Height-for-Age Z-scores, mean (SD)	-1.19 (0.91)	-0.94 (1.20)
Weight-for-Age Z-scores, mean (SD)	-1.44 (0.95)	-1.35 (0.87)
Weight-for-Height z scores, mean (SD)	-0.94 (0.90)	-0.96 (0.79)
BMI (kg/m^2), mean (SD)	14.7 (1.4)	14.7 (1.3)
Parasite density at recruitment (day 0), geometric mean, (95% CI)	47,783 (31,617 - 71120)	32,496 (21,910, - 47,507)
Hb [g/L] at recruitment (Day 0), mean (SD)	103.9 (14.2)	101.5 (14.8)
Hb [g/L] at baseline (Day 3), mean (SD)	93.4 (9.6)	93.7 (10.2)
Iron deficient[1] at baseline (Day 3) (%)	23 (46)	23 (50)

1 *Iron deficiency was defined as Znpp ≥ 61µmol/mol of Hb &, MCV≤ 73fl & MCH ≤25pg*

Table 4a: Predictors of final haemoglobin at day 90 using univariable linear regression

Parameter	Regression coefficient (95%CI)	P-value
Parasite count at recruitment (day 0)	3.5 (1.1, 5.8)	0.004
Sex	-0.4 (-3.3, 2.5)	0.8
Iron deficiency at baseline (day 3)	-12.1 (-14.7, -9.5)	<0.001
Height-for-Age Z-scores (HAZ) <-2	3.2 (1.8, 4.6)	<0.001
Weight-for-Age Z-scores (WAZ) <-2	2.6 (1.0, 4.2)	0.001
Weight-for-Height (WHZ) z scores <-2	0.1 (-1.6, 1.8)	0.9
Age in months	0.4 (0.3, 0.4)	<0.001
Initial anti-malaria treatment arm	1.2 (-2.1, 4.4)	0.5
CQ *vs.* placebo randomisation group	-0.5 (-3.4, 2.5)	0.8
Hb [g/L] at recruitment (day 0)	0.6 (0.5, 0.6)	<0.001
Hb [g/L] at baseline (day 3)	0.7 (0.6, 0.8)	<0.001
Village	1.9 (0.9, 3.0)	<0.001
Presence of sub-microscopic parasitaemia at any time point during follow-up	0.6 (-7.1, 8.2)	0.9
Year of study	-1.3 (-4.4, 1.8)	0.4

Table 4b: Independent predictors of final haemoglobin (day 90) using multivariable linear regression

Parameter	Regression coefficient (95% CI)	P value
Day 0 Hb	0.44 (0.36-0.52)	<0.001
Presence of iron deficiency	-3.0 (-5.57, -0.43)	0.022
Height-for-age Z score <-2	1.48 (0.41, 2.56)	0.007
Age in months	0.21 (0.14, 0.28)	<0.001

Model: Adjusted r^2=0.46, F=76.6, Prob>F <0.001

Figure Legends

Figure 1: CONSORT Flow Diagram describing participants' movements in 2007 & 2008

Figure 2 Mean change in haemoglobin concentration at day 30 and day 90 from baseline at day 3 by treatment group for each year of study and for both years combined.

Error bars represent the 95% confidence intervals

Change in Hemoglobin at day 3 is compared to haemoglobin concentration at presentation with malaria and initiation of anti-malaria treatment. Thereafter the comparison is with baseline levels at day 3 after completion of anti-malarial treatment. Error bars represent the 95% confidence intervals

Figure 3. Percentage of participants with sub-microscopic malaria (qPCR) over time by post-malaria treatment group

Figure 4. Reticulocyte percentage as an indicator of bone marrow response by treatment group for both years combined.

Figure 5. Urinary neopterin by post-malaria treatment group

Randomised controlled trial of weekly chloroquine to re-establish normal erythron iron flux and haemoglobin recovery in post-malarial anaemia

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Sources of support: This study was supported by the UK Medical Research Council (MC-A760-5QX00). The funder had no role in the design, analysis or reporting of this trial.

Running head: Chloroquine in post-malarial anaemia

Abbreviations used: ACT, co-artemether; CQ-SP, chloroquine plus sulphadoxine-pyrimethamine; Hb, haemoglobin; MCH, mean cell haemoglobin; MCV, mean cell volume; RCT, randomised controlled trial; SMA, severe malarial anaemia; ZnPP, zinc protoporphyrin.

Trial registration: The clinical trial registration number is NCT00473837 (ClinicalTrials.gov).

Abstract

Objective: To determine if low-dose weekly chloroquine (CQ) therapy improves recovery from malaria-associated anaemia.

Design: Proof of concept randomised clinical trial.

Setting: West Kiang District, Lower River Region, The Gambia

Participants: Children resident in participating communities, aged 12-72 months with uncomplicated malaria identified using active case detection over two consecutive malaria transmission seasons.

Interventions: In 2007 eligible children were randomised to chloroquine-sulphadoxine/pyrimethamine (CQ-SP) or co-artemether (ACT) anti-malarial therapy, and after parasite clearance on Day 3 subsequently re-randomised (double-blind) to weekly low-dose CQ (5mg/kg) or placebo. In 2008 all eligible children were treated with ACT and subsequently randomised to CQ or placebo.

Outcome measures: The primary outcome was change in haemoglobin from baseline (Day 3 of anti-malarial treatment) to Day 90 in the CQ and placebo treatment arms. Secondary outcomes were changes in urinary neopterin as a marker of macrophage activation, markers of erythropoietic response and prevalence of sub-microscopic parasitaemia. Change in haemoglobin in the placebo arm by initial anti-malarial treatment was also assessed.

Results: In 2007 101 children with uncomplicated malaria were randomised to anti-malarial treatment with CQ-SP or ACT and 65 subsequently randomised to weekly CQ or placebo. In 2008 all children received ACT anti-malarial treatment and 31 subsequently randomised to weekly CQ or placebo. Follow up to Day 90 was 96%. There was no effect of weekly CQ *vs.* placebo on change in haemoglobin at Day 90, (CQ: +10.04 g/L [95% CI:6.66/13.42] *vs.* placebo: +7.61 g/L [95% CI:2.88/12.35]). There was no effect on the secondary outcomes assessed, or effect of initial anti-malarial therapy on haemoglobin recovery. Higher Day 90 haemoglobin independently correlated with older age, not being stunted, higher haemoglobin at Day 0 and adequate iron status at Day 3.

4

Conclusions: Weekly low-dose CQ after effective anti-malarial treatment is not effective in improving recovery from post-malaria anaemia.

5

Article Focus

- To determine if 3 months of low-dose weekly chloroquine (CQ) (5mg/kg) therapy after effective treatment of uncomplicated malaria can improve haemoglobin recovery after malaria-associated anaemia in rural African children.
- To test the hypothesis that chloroquine may enhance haematologic recovery after a malaria episode through its anti-inflammatory and immuno-modulatory actions, anti-macrophageal iron loading effect, and anti-malarial effect (despite widespread resistance).

Key Messages

- Weekly low-dose CQ after effective anti-malarial treatment was not effective in improving recovery from mild to moderate post-malaria anaemia.
- Weekly low dose CQ at 5mg/kg had no clear effects on erythropoietic responses as determined by reticulocyte percentage, or immune-mediated inflammation as assessed by urinary neopterin, a marker of macrophage activation.

Strengths and limitations

- The strengths of this study are its randomised design and double blinding of the allocation to weekly CQ or placebo and that few subjects were lost to follow-up. The continuous follow-up and active case detection for all children ensured that malaria infections were detected early and within similar time-frames, plus resurgent or new clinical infections were excluded from the analysis of haemoglobin change. A strict malaria case definition was adhered to and the measurement of sub-microscopic parasitaemia over time allowed the effects of incomplete treatment to be modelled.
- A limitation of the study was the need to recruit participants over 2 malaria transmission seasons and the significantly reduced malaria transmission rate in 2008 and thus reduced

recruitment -which also appeared to affect the severity of malaria in terms of parasite densities at first detection and haemoglobin concentrations. However, the confidence intervals of the effect size in the final multivariable analysis do not include the clinically significant effect size included in the sample size calculation and thus our observation is unlikely to be due to a type II error as a result of lack of power to detect a true effect.

- It is possible that the relatively low dose of CQ we used was insufficient to mediate the effects that we were testing for and we were unable to conduct pilot studies of CQ drug dosage and duration to determine the potential best treatment regime to test or of the timings of evaluation of possible mechanisms of effect.
- The change in design in 2008 to all children receiving ACT anti-malarial treatment did not affect the analysis of the primary outcome. The change did, however, limit our ability to determine the effect of initial anti-malaria therapy on haemoglobin response in the placebo group. An effect of anti-malarial treatment CQ on Hb, when in the context of adequate parasite clearance as was observed, might have been expected to occur in the early period of recovery.

Introduction

The precise contribution of malaria to anaemia is difficult to calculate mainly because many anaemia-causing conditions co-exist in children living in malaria-endemic countries. [1] Murphy and Breman [2] estimate the annual incidence of severe malaria anaemia (SMA) to be 1.42 to 5.66 million resulting in between 194,000 to 974,000 deaths in children younger than five years. SMA increases the risk of death in children with malaria [3], many of whom die shortly after admission before blood transfusion can be commenced [4 5] and after discharge in the community [6]. In addition, blood transfusion, the standard treatment for severe malaria anaemia exposes the child to the risk of transfusion-related infections like Human Immunodeficiency Virus (HIV) and hepatitis [7]. Among a nationally representative sample of children aged one to five years in The Gambia in 1999, 76% had a haemoglobin (Hb) less than 11g/dl and 15% had Hb less than 7g/dl [8]. It is likely that most of the cases of moderate to severe anaemia were due in great part to malaria [9-11]. A better understanding of the pathogenesis of malaria anaemia is required to improve its prevention and management.

Multiple immune and non-immune-mediated mechanisms cause anaemia during and after a malaria episode[12] including the destruction of non-parasitized erythrocytes which may account for more than 90% of erythrocyte loss[13 14]. One important component is inflammation-induced iron delocalisation which is characterised by sequestration of iron by the monocyte-macrophage system and hepatocytes, resulting in a state of pseudo-iron deficiency; a state that reverses after the inflammation has subsided[12]. The trans-membrane protein ferroportin is the exclusive route for cellular iron efflux and is responsible for releasing absorbed iron from enterocytes and sequestered iron from macrophages[12 15 16]. Hepcidin, the master-regulator of iron metabolism which responds both to iron status and inflammation[15], blocks ferroportin. Clinically, malaria-induced inflammation has been shown to be associated with a hepcidin-mediated reduction in intestinal absorption of iron. [17] Inflammatory cytokines, particularly IL-6 and IL-1 stimulate hepcidin release[18 19] resulting both in

reduced iron absorption of dietary iron and impaired macrophageal release of iron; the latter effect being quantitatively much more significant as macrophageal iron recycling and transport to the erythron is 25-30 fold greater than the influx of dietary iron. In this way hepcidin is the mediator of the anaemia of chronic disease[15]. TNF-α could also play a role in ferroportin-mediated iron transfer independent of hepcidin[20]. Rapid resolution of malaria-induced inflammation could therefore limit the degree and duration of malaria anaemia by reducing the duration of iron delocalisation.

Chloroquine is a cheap and safe antimalarial with proven anti-inflammatory properties; and has been shown to reduce iron delocalisation *in vitro*[21,22]. We hypothesised that chloroquine may enhance haematologic recovery after a malaria episode through its anti-inflammatory and immuno-modulatory actions, anti-macrophageal iron loading effect, and anti-malarial effect (despite widespread resistance). We designed a proof of concept study to investigate the potential use of chloroquine in the management of mild post malaria anaemia.

Materials and Methods

Study Design and population

This study was a population-based randomised, controlled trial (RCT) in children aged 12 to 72 months conducted over two malaria seasons in 2007 and 2008 in West Kiang District, Lower River Region, The Gambia. The study was initially designed and conducted as a 2x2 randomised placebo-controlled trial in 2007 but had to be changed, due to changes in national malaria treatment guidelines, to a two-arm RCT during the 2nd malaria transmission season in 2008. In the 2x2 phase of the study, children with malaria were initially openly randomised to receive a therapeutic dose regime of chloroquine plus sulphadoxine-pyrimethamine (CQ-SP) or co-artemether (ACT) (Day 0). After treatment, on Day 3, children were subsequently randomised (double blind) to weekly chloroquine or placebo until Day 90 (**Figure 1**). In the two-arm phase, all enrolled children with malaria were treated with ACT and then randomised and followed up as before (**Figure 1**). A combination of active and passive case detection was used to identify children in participating communities with malaria using a standard case definition of clinical malaria (see below).

The climate of this district is typical of sub-Sahelian Africa with a long dry season lasting from November to June and a relatively short rainy season lasting from July to October. Malaria transmission is seasonal, hyperendemic and follows the rainy season, occurring between July and December. The prevalence of children experiencing one or more malaria episodes among a cohort of 1002 children aged between 1-6 years under active malaria surveillance during a population-based study in 2003 was 34% (Doherty et al. Unpublished data). During the study period standard malaria control activities in the district consisted of insecticide treated bednet distribution and case finding and treatment. In 2008 the standard first-line antimalarial treatment regimen for uncomplicated malaria in The Gambia was changed from CQ-SP to co-artemether.

Inclusion & exclusion criteria

Eligibility for malaria surveillance and subsequent possible enrolment into the RCT included all children in the participating communities, aged between 12 and 72 months at the start of the surveillance period and who were not severely wasted, defined as weight-for-height Z scores less than -3SD.

Inclusion criteria for enrolment included: 1) uncomplicated malaria at Day 0, defined as a history of fever 48 hours prior to presentation or a measured temperature of 37.5°C or more with peripheral parasitaemia (asexual forms of *P. falciparum* ≥500/µL by microscopy) on Day 0 of the study; and 2) for randomisation to the post-malaria intervention, children had to have a Hb between 69g/L - 110g/L on Day 3 with no peripheral parasitaemia.

Children were excluded from the RCT if they were: 1) unable to take oral medications; 2) had features of severe malaria;[1] 3) had a known haemoglobinopathy; 4) were enrolled in another project; 5) had already received antimalarial drugs from other sources outside the project team; or 6) were prescribed other drugs with potential antimalarial or anti-anaemic effects such as co-trimoxazole and haematinics.

Following randomisation to weekly CQ or placebo children were withdrawn from the study if: 1) they developed a second malaria episode or had life-threatening symptoms, more severe disease or serious adverse reactions; 2) left the study area for more than 14 days; 3) received antimalarials from other sources; or 4) Hb fell below 70g/L.

Field and clinical procedures

To effect the surveillance protocol, the study villages were divided into geographically adjacent clusters supervised by a resident study nurse who visited each village morning and evening and provided 24-hour clinical coverage to all enrolled children in their cluster. The active surveillance consisted of twice weekly temperature monitoring of enrolled children by village assistants; while the passive surveillance consisted of mothers referring sick children to the visiting study nurse. Children with fever – reported or measured – were screened for malaria using a rapid diagnostic test (MRDT) (Cortez diagnostics, INC, USA) after clinical evaluation. Children testing negative to the

MRDT were treated based on the likely diagnosis from the symptoms and signs. Children testing positive to the MRDT were commenced on antimalarials immediately (either CQ-SP or ACT in 2007, and ACT in 2008 for three days) and directly observed by the study nurse and managed as appropriate. Finger prick blood samples (days 7, 15, 30 & 70) were collected in an EDTA microtainer while venous blood (2mls – days 0 & 90; and 5mls – days 3 & 45) samples were collected in an EDTA vacutainer and sent to the study laboratory on the same day. Co-morbidities were treated as appropriate, avoiding co-trimoxazole and haematinics. For the subsequent visits after initial malaria diagnosis at Day 0, children were seen by the study nurse and blood samples and clinical data collected as per protocol. Only children who fulfilled the inclusion criteria for advancement to the weekly chloroquine/weekly placebo phase of the study were sampled after Day 3. These children were also followed up on days 15, 30, 45, 70 (except in 2008) and 90.

Randomisation and blinding

The randomisation to malaria treatment group in 2007 (CQ-SP or ACT) was open (non-blinded to trial staff, investigators or participants) and was done by sequential assignment as children were identified. The block randomisation to the post-malaria treatment of weekly CQ or placebo in both 2007 and 2008 was double blinded and was carried out in blocks of eight. The randomisation codes were generated by a staff member independent of the study team and held by the external trial monitor. Treatment codes were labelled A to H and placed in sequentially numbered, opaque sealed envelopes held by the study nurses. Allocation to the treatment was by matching the code in the envelope to a bottle of the intervention labelled with the same code and then labelled with the subject ID.

Interventions

For children openly randomised to malaria treatment at Day 0 with CQ-SP, children received choroquine syrup (Chlormal®, Medreich Sterilab Ltd, Avalahalli, Bangalore, India) consisting of 50mg chloroquine base per 5mls as chloroquine phosphate at a dose of 10mg/kg for 3 days (days 0-2). SP (Micro Laboratories, India) was co-administered as a 525 mg tablet (500mg sulfadoxine and 25mg

pyrimethamine) on Day 0 only at a dose of ½ tablet/10kg. Children who were randomised to ACT treatment in 2007 and all children in 2008 received co-artemether (Coartem®) tablets (Beijing Novartis Pharma Ltd, Beijing China for Novartis Pharma AG, Basle, Switzerland) as blister packets of 6 tablets per card with each tablet containing 20mg artemether and 120mg lumefantrine. The treatment dose comprised a stat dose on Day 0 and then 8 hours later, followed by twice daily doses for the next two days with doses depending on body weight (5 - <15 kg – one tablet/dose; 15 - <25kg – 2 tablets/dose; 25 - <35kg – 3 tablets per dose).

During the second blinded phase of the trial children received either weekly CQ syrup (as above) but at a dose of 5mg/kg or matching placebo syrup (prepared by Royal Victoria Teaching Hospital pharmaceutical laboratory in collaboration with Atlantic Pharmaceuticals Limited, Latrikunda Sabiji, The Gambia) The syrups were in similar amber coloured bottles with matching caps and labels. Doses were administered in the child's home under direct observation by the study nurses who waited for at least 10 minutes after administering the drugs to ensure that they were not vomited.

Primary and secondary outcomes

The primary outcome measure was Hb change from Day 3 post treatment of malaria episode to Day 90 in the weekly CQ and placebo arms.

The secondary outcomes were to compare between the weekly CQ and placebo arms: (1-i) changes in erythropoietic response between Day 3 and Day 30; and (1-ii) between day 3 and day 90; (2) changes in urinary neopterin from Day 3 to Day 30 as a marker of macrophage activation; (3) prevalence of sub-microscopic malaria parasitaemia using PCR at each time point, and; (4) Hb change from Day 3 to Day 90 in the two placebo arms to investigate the effects of initial malaria treatment therapy.

Sample size

Comment [tony1]: Is this necessary … at least it is not a post hoc estimate!

During initial study design we used a working assumption of a standard deviation (SD) for change in Hb of 15 g/l[23]. With power 80% and significance level set at 5% we estimated the sample sizes required to detect varying effect sizes for the primary outcome. A sample size of 65 per arm was estimated to be able to detect an effect size of 7.5g/l. This sample size was thought to be achievable and the effect size of likely public health significance.

Laboratory procedures:

Table 1 describes the different laboratory analysis conducted. Zinc protoporphyrin (ZnPP) as a marker of iron status was measured on fresh whole blood using a fluorometer (AVIV Biomedicals, Lakewood, NJ, USA). Full blood count (FBC), haemoglobin and automated reticulocyte count were performed using a Cell Dyne 3700 (Abbott Laboratories. Abbott Park, Illinois, USA). Iron deficiency was defined using 3 parameters; mean cell volume (MCV), mean cell haemoglobin (MCH) and ZnPP. The cut off values for the different parameters employed were: ≥61µmol/mol of Hb for ZnPP [24], ≤73fl for MCV [25] and ≤25pg for MCH [25]. Combining these parameters has been shown to increase their sensitivity and specificity to identify iron deficiency [26]. Malaria parasites were identified in a thick peripheral blood smear stained with Giemsa overnight and read by light microscope with x100 magnification in oil immersion. Parasite DNA extraction and subsequent quantitative PCR amplification were carried out using established protocols [27]. In brief, DNA extraction was done using the QIamp DNA Mini Kit (Qiagen™ Ltd. UK) according to the manufacturer's instructions with minor modifications. Extracted DNA samples were stored at -20°C. Quantification of parasite density was done with a TaqMan assay employing a fluorogenic probe to detect specific parasite products and using an Opticon® 2 Real Time PCR machine and Opticon™ version 3.1 software (Bio-Rad Laboratories, Inc., USA). Primers and probes were synthesized by Applied Biosystems, UK. Urinary neopterin levels were measured using an ELISA technique (BRAHMS Neopterin EIA, B.R.A.H.M.S, Germany). The stored urine was allowed to thaw at room temperature and then diluted 1:100 and the rest of the procedure was according to the manufacturer's instructions.

Statistical analysis:

Delta haemoglobin sSummary statistics are presented as means and standard deviations (SD) for normally distributed continuous variables, 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 are presented for log-transformed variables. Two-sided student's t tests were used to compare results between two treatment arms.

Visual inspection of Hb change between baseline at Day 3 and later time points by treatment arm suggested that by Day 30, Hb change in both treatment arms had reached a plateau. ~~Therefore Hb data from Day 30 to Day 90 were pooled for subsequent analysis. This pooled Hb is referred to as 'final Hb'.~~ To fully determine the effect of treatment group on Hb change we used random effects models using the repeat measures at day 30 and day 90. Initial malaria treatment group (CQ-SP vs ACT), Hb at recruitment (Day 0 Hb), baseline Hb (Day 3), age in months, year of study, and the child's village were included in models "*a priori*" and hence adjusted for.

Linear regression was used to investigate further potential predictors of Hb change in the study population. First, 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 to assess confounding. The final regression model consisted of only the variables that maintained a significant association with Hb in the multiple regression models. All analyses were carried out using STATA version 8 (StataCorp, College Station, TX, USA).

Ethical clearance

The Scientific Coordinating Committee of the Medical Research Council (UK), The Gambia, and the MRC/Gambia Government Joint Ethical Committee approved the study (SCC 1076). Written informed consent was obtained from parents/guardians of the children prior to enrolment. The clinical trial registration number is NCT00473837 (ClinicalTrials.gov).

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Results

Characteristics of the study population under malaria surveillance

Figure 1 describes the flow of participants and reasons for exclusions in the study. In 2007, 1445 of 1817 (80%) potentially eligible children identified through the Keneba MRC DSS database from 14 villages were included in the malaria surveillance. In 2008, 1220 of the 1413 (86%) identified children from 5 of the original villages were included in the malaria surveillance (smaller villages were excluded as they had contributed few cases in 2007). **Table 2** describes the characteristics of the children placed under surveillance for malaria. The proportion of children with a positive malaria rapid test during surveillance (only first event per child counted) fell from 7.3% (105/1445) in 2007 to 4% (49/1220) in 2008. In the first year of the study, 101 (96%) of the children with a positive malaria rapid test were randomised to CQ-SP or ACT treatment, and 65 (64%) subsequently randomised at Day 3 to weekly chloroquine or placebo, with all children completing follow up until Day 30 and 33/34 completing follow-up until Day 90 in the weekly CQ and 28/31 in the weekly placebo group. In the second year of the study 31 of the 49 (63%) cases with a first positive malaria rapid test were subsequently randomised to weekly CQ or placebo after treatment with ACT, all of whom completed follow up until Day 90 (16 CQ & 15 placebo). ~~After determining a lack of change between Day 30 and Day 90 Hb measurements across all treatment groups it was decided to include the 4 children who were excluded after Day 30 of follow-up due to development of a second malarial episode, in the analysis of "final Hb" by use of Hb at Day 30~~. Thus 96 children were included in the analysis of the primary and secondary outcomes, except for the analysis of effect of initial malaria treatment within the placebo arms which included 31 children.

Table 3 describes the characteristics of participants randomised to weekly chloroquine and weekly placebo for both years combined. Demographic and anthropometric characteristics were similar between the groups as was baseline Hb at Day 3. The prevalence of iron deficiency measured at baseline was 43.1% (28/65) in 2007 and 58.1% (18/31) in 2008 with a similar prevalence between

the CQ/placebo groups within each year (data not shown) and in both years combined. However parasite density was lower in the placebo group (**Table 3**). No adverse events were reported in either treatment group.

Effect of weekly CQ on haemoglobin change during follow-up

To assess the effect of weekly chloroquine on Hb recovery after clearance of peripheral parasitaemia, we compared change in Hb from Day 3 to Day 90 and Day 3 to Day 30 in the two arms. No significant differences in Hb change between the two groups were observed, either for the data ~~pooled~~ for both study years or for each study year separately (both years combined: change in Hb at Day 90, CQ: +10.04 g/L [95% CI:6.66, 13.42] *vs.* placebo: +7.61 g/L [95% CI:2.88, 12.35]) (**Figure 2**). Thus the effect size of the CQ treatment was 2.43 g/L [95& CI -3.38, 8.24] compared to the 7.5g/L we included in the sample size calculation. However, at Day 15, children receiving weekly CQ had an increase of 3.5 g/L (95% CI: 0.5, 6.4) compared to 0.8g/L (95% CI: -2.3, 3.9) in children in the placebo arm, but this difference did not reach statistical significance (p=0.2). The lack of effect of ~~The~~ weekly CQ remained ~~overall result was unchanged~~ when adjusting for factors determined '*a priori*' (Hb at recruitment (Day 0), Hb at baseline (Day 3), age (in months), year of study, initial treatment received (CQ-SP *vs* ACT), and village group) using a random effects repeated measures model including Hb measured at day 30 and day 90 (coefficient =1.43 [95% CI: -1.70, 4.57). Furthermore, adding parasite density at recruitment, presence of sub-microscopic parasitaemia at any time point during follow-up, and presence of iron deficiency had no effect on the estimates of effect for CQ *vs*. placebo treatment during follow-up (data not shown).

Predictors of haemoglobin at Day 90

In the absence of an effect of weekly CQ on Hb change after successful malaria treatment, we assessed what other factors might determine final Hb. Using simple linear regression significant positive associations were observed for parasite density at Day 0, better nutritional status, age, Hb at Day 0 at malaria diagnosis and Day 3 of successful treatment. Presence of iron deficiency at baseline was associated with significantly lower final Hb. There was no apparent effect of initial

malaria treatment (CQ-SP *vs.* ACT) (**Table 4a**). In a final multiple regression model, Hb at Day 0 was the strongest predictor of final Hb and other independent predictors of final haemoglobin were presence of iron deficiency at Day 3, degree of stunting (height-for-age Z score), and age (in months). Children with higher Hb at diagnosis and older children had greater final Hb. Children with iron deficiency at Day 3 had significantly lower final Hb with a mean difference of 12.1g/L (**Table 4b**).

Effect of initial anti-malaria treatment (CQ-SP *vs* ACT) on haemoglobin change

A secondary outcome of this study was Hb change from Day 3 to Day 30 and Day 90 in the placebo arm to determine whether initial anti-malaria treatment (CQ-SP or ACT) treatment affected macrophage iron loading in acute clinical malaria and its subsequent release. As indicated in the analysis of Hb at Day 90 there was no significant effect of inital anti-malarial treatment. Please see Supplementary **Figure 2** depicting Hb change during follow up from Day 0 to Day 90 in the placebo arm by initial antimalarial treatment received and the accompanying text.

Sub-microscopic parasitaemia, inflammation, bone marrow response, haemoglobin change and treatment groups

Sub-microscopic parasitaemia was commonest in the first seven days of acute phase follow up (days 3-15). By Day 30, most were negative (77%). The prevalence of sub-microscopic parasitaemia at days 30 and 90 combined was 15.1% and there was no evidence of an effect of post-malaria treatment group or of initial anti-malarial treatment (**Figure 3 & Supplementary Figure 2~~4b~~**). Most of the sub-microscopic parasitaemia at these time points was of a very low level (one to two parasites per microliter). However, higher parasite density at diagnosis was associated with an increased likelihood of subsequent sub-microscopic parasitaemia at day 30 or 90 with a one log increase in parasite count increasing the odds of having sub-microscopic parasitaemia by 1.6 (P=0.005). Although the presence/absence of sub-microscopic parasitaemia during follow up (days 30 & 90) was not associated with final Hb (**Table 4**), in children with high qPCR (quantitative PCR parasite count) parasite count (> 500/µL, N=3) at Day 30, Hb at Day 30 was significantly lower compared to children with one to two parasites/µL or none at Day 30 (82.3g/L vs 102.8g/L; p = 0.01).

Similarly, the mean Hb change was also reduced in those with persistent high parasitaemia 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). Bone marrow response as assessed by percentage of reticulocytes peaked at Day 15 (4.5% in both treatment groups combined), compared to similar levels of around 2% at all other measured time points (Days 3, 7, 15 & 90). There was no evidence of an effect of either post-malaria treatment group (**Figure 4**) or of initial anti-malarial therapy (data not shown)~~(**Figure 5)**~~ on bone marrow response ~~(data not shown)~~. Urinary neopterin, a product of activated macrophages and therefore a marker of a pro-inflammatory immune status, was strongly associated with qPCR parasite count at Day 30 (regression coefficient: 0.2 (95%CI: 0.08, 0.33), p = 0.003; adjusted R^2 = 0.4091) and was also associated with Hb at Day 30. There was no evidence of an effect of post-malaria treatment group (**Figure 5)** or of initial anti-malarial treatment on neopterin concentrations (**Figure S3**).

Discussion

Chloroquine is used in the treatment of several diseases in addition to its antimalarial use[28] [29]. However, we believe this to be the first study to assess its potential role in the management of post-malaria anaemia. We hypothesised that since malarial anaemia is essentially a systemic inflammatory response to infection[12], chloroquine, by suppressing plasmodium-induced inflammation, could enhance erythropoietic recovery. We tested this hypothesis by comparing two groups of children – one group receiving weekly chloroquine and the other group weekly placebo after complete clearance of peripheral parasitaemia as determined by microscopy. The primary outcome measure was the recovery in Hb at Days 30 and 90 compared to Day 3 Hb levels. Our results showed no significant difference in the Hb change between the two groups. The data was interrogated further by a random effects model modelling data at Day 30 and Day 90 simultaneously whilst adjusting for Hb at Day 3 and taking into account random measurement errors and missing data. This confirmed that weekly chloroquine at 5mg/kg body weight did not improve Hb recovery after uncomplicated clinical malaria. There was a suggestion that weekly CQ may have increased the rate of recovery in Hb change at Day 15 accompanied by non-signficantly increased reticulocyte percentage.

A limitation of the study was the need to recruit participants over 2 malaria transmission seasons. In 2008 there was a reduced malaria transmission rate and thus fewer cases available for recruitment and also an apparent effect on the severity of malaria in terms of parasite densities at first detection and effect on haemoglobin concentrations, with a significantly higher baseline Hb in 2008. However, the change in design that was necessitated in 2008 did not change the primary outcome analysis. Furthermore, the confidence intervals of the observed effect size in the final multivariable analysis do not include the effect size included in the sample size calculation and thus our observation is unlikely to be due to a type II error as a result of lack of power to detect a true effect of this magnitude. ~~.~~

The change in design in 2008 to all children receiving ACT anti-malarial treatment did not affect the analysis of the primary outcome. The change did, however, limit our ability to determine the effect of initial anti-malaria therapy on haemoglobin response in the placebo group. An effect of anti-malarial treatment CQ on Hb, when in the context of adequate parasite clearance as was observed, might have been expected to occur in the early period of recovery.

The recommended anti-inflammatory dose of CQ is 3-4mg/kg/day. However, due to lack of safety data for this condition and considering potential adverse effects, particularly retinal damage, we used the lower prophylactic dose of 5mg/kg/week. Using this low dose might have contributed to our inability to detect an effect of CQ. Drenou et al [30] reported the successful control of sideroblastic anaemia (a condition of reduced incorporation of iron into haem in the presence of sufficient or high iron) was for six years on 100mg daily chloroquine without need for blood transfusion.

We had hypothesized that chloroquine will impede macrophageal iron sequestration during the acute clinical episode and improve iron availability for the process of erythropoiesis. Urinary excretion of neopterin is markedly increased in individuals infected with malaria, and corresponds to concurrent activation of T cells and macrophages[31-33]. We measured the urinary neopterin at Days 3, 15 and 30 and expected to see a more rapid decline in neopterin level in the weekly chloroquine group than in the weekly placebo group; but no difference between the treatment groups was observed. It is possible that a difference may have occurred at an intermediate time point between Day 3 and Day 15, when levels had already almost normalised. Indeed, Brown et al [33] 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 our study, this would coincide with study Days 0 to 3. In future, it might be more informative to carry out more measurements during the first week of malaria diagnosis.

The participants were followed up for 90 days because reports had suggested that Hb continues to fall even after successful malaria treatment primarily due to continuing inflammation[34]

[35]. In this study we found no evidence of persistent inflammation, except in the few cases with remaining parasitaemia at Day 30, who also had decreased absolute Hb and Hb change at Day 30 compared with children with no parasitaemia or parasite counts of 1-2/microlitre.

Our observation that children with higher Hb at diagnosis had greater Hb recovery is potentially paradoxical as one would have expected the reverse through statistical regression to the mean and greater 'catch up' in children with lower starting Hb at Day 3. However, as the majority of iron used for Hb recovery is recycled[24], those with higher Hb at Day 0 have more iron available for recovery. Our non-provision of exogenous iron during the study may have restricted the ability of the more anaemic children to respond appropriately as suggested by our observation of reduced Hb recovery in children with apparent iron deficiency at Day 0. An earlier study in Gambian children showed improved haematological recovery in children treated for malaria and co-supplemented with iron compared to placebo[36]. However, we have also shown in Gambian children reduced iron absorption from supplementation in post-malarial anaemia, whilst also experiencing greater Hb recovery compared to supplemented children with iron-deficiency anaemia. Thus causing us to question the efficacy of iron supplementation given within 2 weeks of anti-malarial treatment[24]. Without the inclusion of a further randomised treatment arm of post-malarial treatment iron supplementation it is not possible to make further recommendations concerning the recent argument for the provision of iron supplementation to children in malaria endemic areas with regular malaria surveillance and treatment facilities[37].

Our finding that stunted children recovered Hb poorly is consistent with an earlier report of stunting being a determinant of the severity of malaria-associated anaemia[38]. In our population this effect was independent of iron status.

Age has been shown to be strongly associated with risk of severe anaemia in children with acute malaria; the risk decreasing with increasing age[39]. In addition, we have shown in this study that older children recover better from anaemia after an acute malaria episode than younger children.

Paying special attention to younger children with malaria anaemia could reduce malaria related mortality in children.

In conclusion, weekly chloroquine administered for three months to children with mild malaria anaemia at a dose of 5mg/kg/week resulted in no improvement in erythropoietic recovery compared to placebo. Older children, children with higher initial Hb, less stunting at diagnosis, and without iron deficiency had better Hb recovery post-malaria. Similar to other reports in the literature, urinary neopterin was elevated during acute malaria. The role of iron deficiency in erythropoietic recovery post malaria noted in this study suggests areas for further detailed investigation of the clinical value in administering both antimalarial and iron supplementation to children with malaria anaemia residing in areas of high iron deficiency and malaria burden.

Acknowledgements

The authors wish to acknowledge the contribution of all the members of the field team – Kabiru Ceesay, Edrisa Sinjanka, Lamin Ceesay, Lamin Sowe, Kalilu Sanneh, Bakary Sarr, Muhammed Sidibeh, Yahya Sanyang, the data staff especially Jahid Hassan and Musa Jarjou, the laboratory staff especially Musa Colley, Karamo Camara, Mustapha Bittaye, and Ebrimah Danso, all the drivers of the MRC Keneba, Ms Cheryl Eniyou O, Mrs Fatou Noho, Ms Jankey Jagne, all the village assistants, and the study subjects and their parents.

Authorship contributions

CVN, CPD and AMP conceived the study with input from SEC. CVN led all practical aspects of the work with logistic and funding support from SEM and AMP. CVN, SEC and AJF were responsible for the statistical analysis and interpretation. CVN and SEC drafted the paper with input and approval from all authors.

Disclosure of Conflicts of Interest

The authors have no conflict of interest.

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TABLES

Table 1: Laboratory investigations conducted

Investigations	Specimen	Days collected[†]	Purpose
Microscopy for malaria parasite	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess parasitaemia
Erythrocyte zinc protoporphyrin	Blood	Days 0 & 3	To assess iron status
Full blood count	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess Hb change over time
Automated reticulocyte percentage	Blood	Days 3, 7, 15, 30, 45, 70, 90	To monitor bone marrow response
Quantitative PCR for *P.falciparum* parasites	Blood	Days 0, 3, 7, 15, 30, 45, 70, 90	To assess sub-microscopic parasitaemia
Urinary neopterin concentration	Urine	Days 3, 15, 30	To assess macrophage activation as measure of immune mediated inflammation

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

Table 2: Baseline characteristics of all participants under surveillance

	2007	2008	Combined
	N=1445	N=1220	N=2665
Mean Age in months (SD)	42.2 (17.2)	40.7 (17.7)	41.5 (17.5)
Mean Weight-for-Height Z-scores (SD)	-1.06 (0.84)	-1.02 (0.85)	-1.04 (0.84)
Mean Weight-for-Age Z-scores (SD)	-1.48 (0.97)	-1.50 (0.94)	-1.49 (0.95)
Mean Height-for-Age Z-scores (SD)	-1.06 (1.21)	-1.17 (1.13)	-1.11 (1.17)
Mean Body Mass Index (kg/m^2) (SD)	14.6 (1.3)	14.7 (1.4)	14.6 (1.4)
Prevalence of a malaria rapid test positive result during surveillance*	105 (7.3)	49 (4.0)	154 (5.8)

*****only the first positive result is counted.*

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Table 3: Baseline characteristics of children randomised to weekly chloroquine and weekly placebo in 2007 and 2008, pooled

Parameter	Chloroquine group N=50	Placebo group N=46
Male sex (%)	28 (56.0)	24 (52.2)
Age in months, mean (SD)	41.9 (18.1)	38.7 (17.8)
Height-for-Age Z-scores, mean (SD)	-1.19 (0.91)	-0.94 (1.20)
Weight-for-Age Z-scores, mean (SD)	-1.44 (0.95)	-1.35 (0.87)
Weight-for-Height z scores, mean (SD)	-0.94 (0.90)	-0.96 (0.79)
BMI (kg/m^2), mean (SD)	14.7 (1.4)	14.7 (1.3)
Parasite density at recruitment (day 0), geometric mean, (95% CI)	47,783 (31,617 - 71120)	32,496 (21,910, - 47,507)
Hb [g/L] at recruitment (Day 0), mean (SD)	103.9 (14.2)	101.5 (14.8)
Hb [g/L] at baseline (Day 3), mean (SD)	93.4 (9.6)	93.7 (10.2)
Iron deficient[1] at baseline (Day 3) (%)	23 (46)	23 (50)

1 *Iron deficiency was defined as Znpp ≥ 61µmol/mol of Hb &, MCV≤ 73fl & MCH ≤25pg*

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Table 4a: Predictors of final haemoglobin at day 90 using univariable linear regression

Parameter	Regression coefficient (95%CI)	P-value
Parasite count at recruitment (day 0)	3.5 (1.1, 5.8)	0.004
Sex	-0.4 (-3.3, 2.5)	0.8
Iron deficiency at baseline (day 3)	-12.1 (-14.7, -9.5)	<0.001
Height-for-Age Z-scores (HAZ) <-2	3.2 (1.8, 4.6)	<0.001
Weight-for-Age Z-scores (WAZ) <-2	2.6 (1.0, 4.2)	0.001
Weight-for-Height (WHZ) z scores <-2	0.1 (-1.6, 1.8)	0.9
Age in months	0.4 (0.3, 0.4)	<0.001
Initial anti-malaria treatment arm	1.2 (-2.1, 4.4)	0.5
CQ *vs.* placebo randomisation group	-0.5 (-3.4, 2.5)	0.8
Hb [g/L] at recruitment (day 0)	0.6 (0.5, 0.6)	<0.001
Hb [g/L] at baseline (day 3)	0.7 (0.6, 0.8)	<0.001
Village	1.9 (0.9, 3.0)	<0.001
Presence of sub-microscopic parasitaemia at any time point during follow-up	0.6 (-7.1, 8.2)	0.9
Year of study	-1.3 (-4.4, 1.8)	0.4

Table 4b: Independent predictors of final haemoglobin (day 90) using multivariable linear regression

Parameter	Regression coefficient (95% CI)	P value
Day 0 Hb	0.44 (0.36-0.52)	<0.001
Presence of iron deficiency	-3.0 (-5.57, -0.43)	0.022
Height-for-age Z score <-2	1.48 (0.41, 2.56)	0.007
Age in months	0.21 (0.14, 0.28)	<0.001

Model: Adjusted r^2=0.46, F=76.6, Prob>F <0.001

Figure Legends

Figure 1: CONSORT Flow Diagram describing participants' movements in 2007 & 2008

Figure 2 Mean change in haemoglobin concentration at day 30 and day 90 from baseline at day 3 by treatment group for each year of study and for both years combined.

Error bars represent the 95% confidence intervals

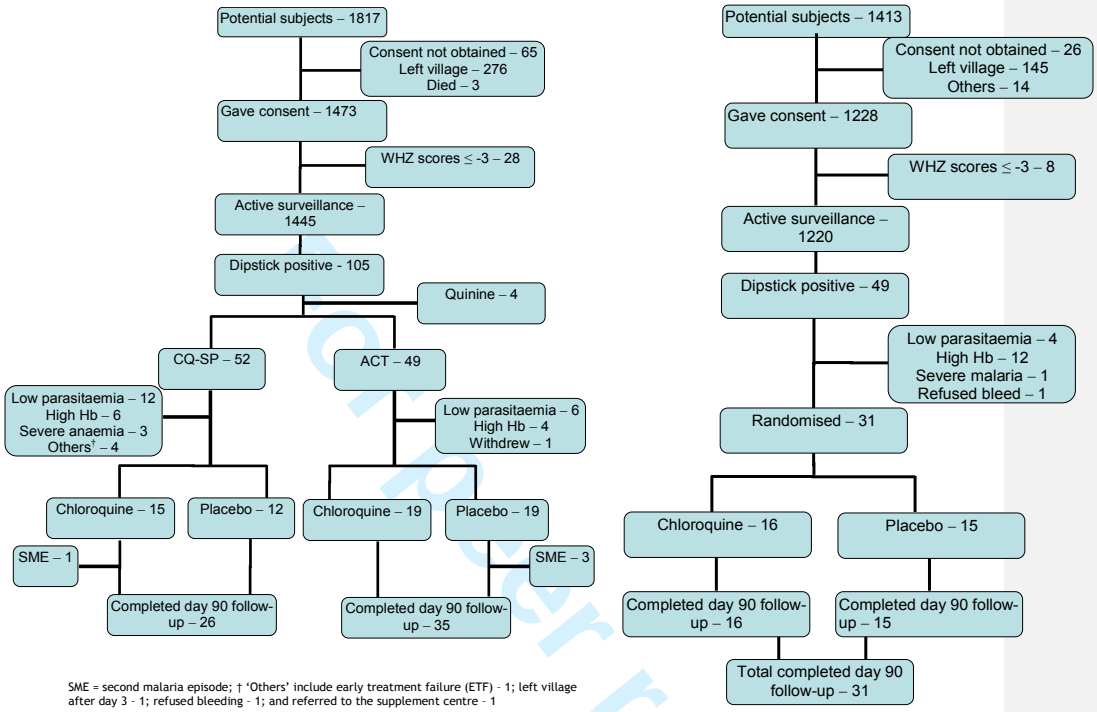
Change in Hemoglobin at day 3 is compared to haemoglobin concentration at presentation with malaria and initiation of anti-malaria treatment. Thereafter the comparison is with baseline levels at day 3 after completion of anti-malarial treatment. Error bars represent the 95% confidence intervals

Figure 3. Percentage of participants with sub-microscopic malaria (qPCR) over time by post-malaria treatment group

Figure 4. Reticulocyte percentage as an indicator of bone marrow response by treatment group for both years combined.

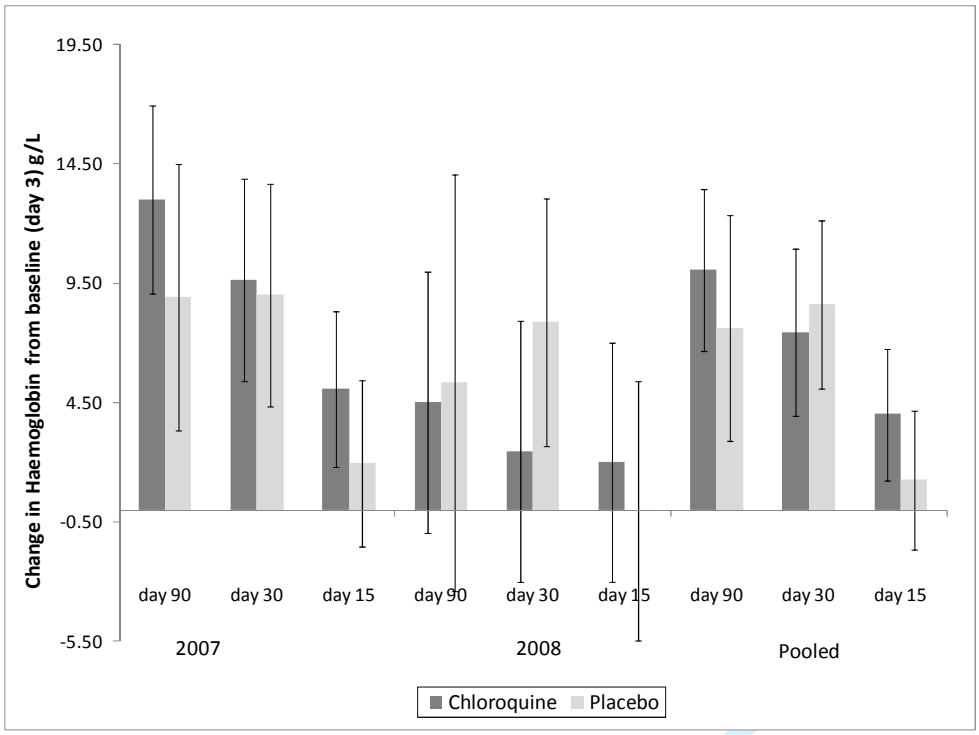
Figure 5. Urinary neopterin by post-malaria treatment group

Figure 1 CONSORT Flow Diagram describing participants' movements in 2007 & 2008



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 2 Mean change in haemoglobin concentration at day 30 and day 90 from baseline at day 3 by treatment group for each year of study and for both years combined.



Error bars represent the 95% confidence intervals

Figure 3. Percentage of participants with sub-microscopic malaria (qPCR) over time by post-malaria treatment group

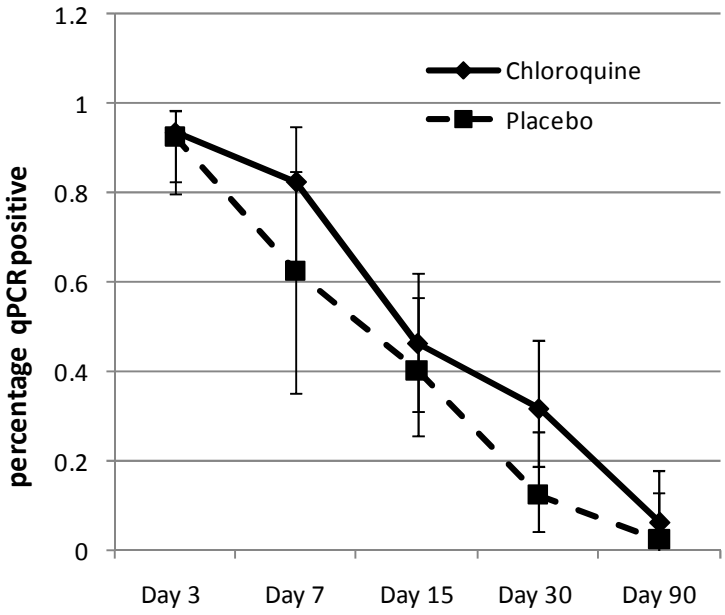


Figure 4. Reticulocyte percentage as an indicator of bone marrow response by treatment group for both years combined.

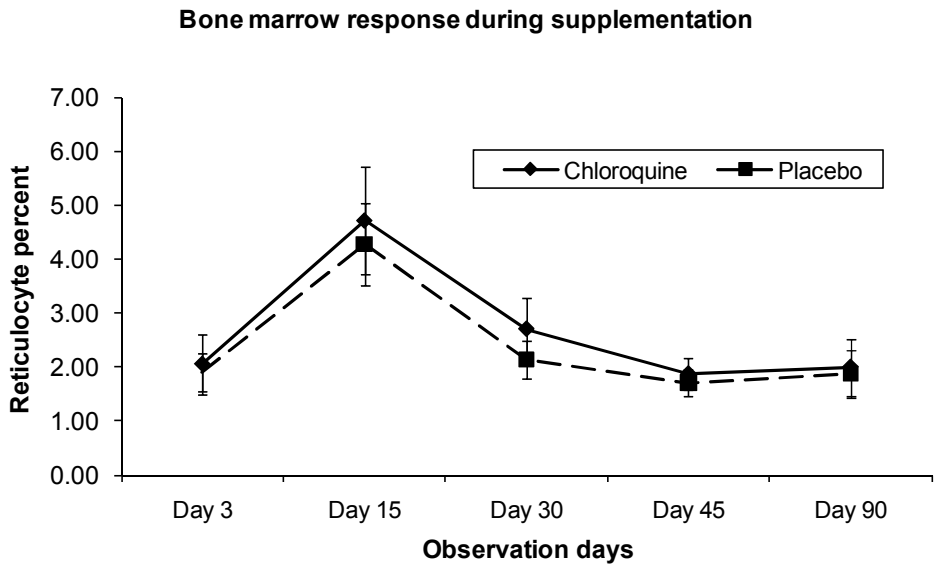


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Figure 5. Box and whisker plot of urinary neopterin by post-malaria treatment group

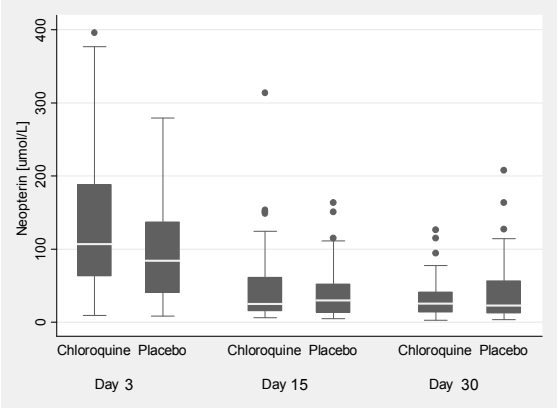

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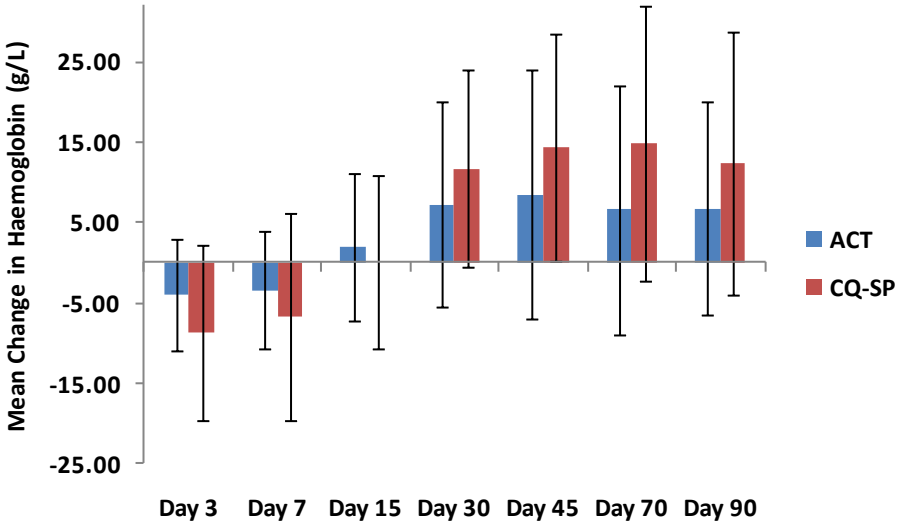
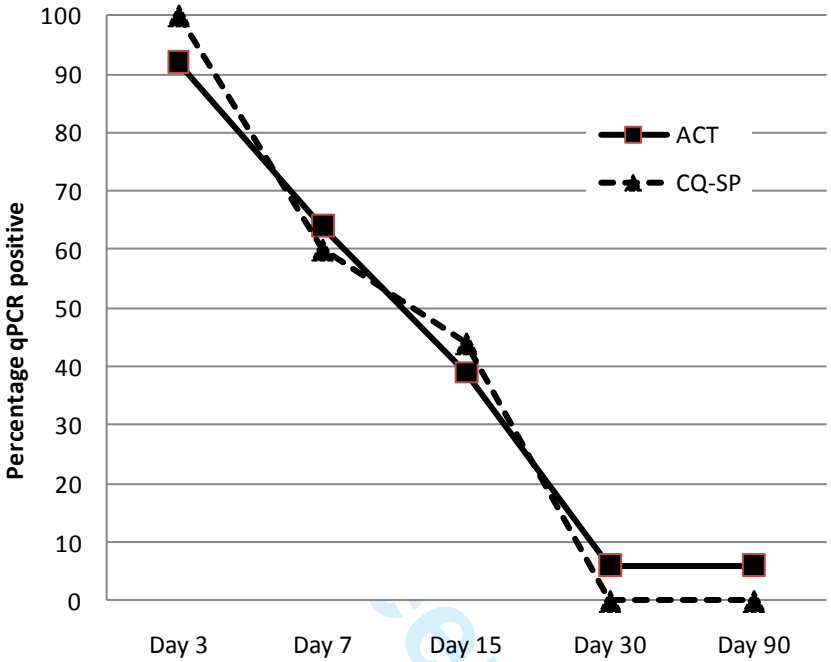
Figure S1 Mean change in hemoglobin in the placebo arm by initial anti-malarial treatment group in 2007 study.



Change in Hemoglobin at day 3 is compared to haemoglobin concentration at presentation with malaria and initiation of anti-malaria treatment. Thereafter the comparison is with baseline levels at day 3 after completion of anti-malarial treatment. Error bars represent the 95% confidence intervals

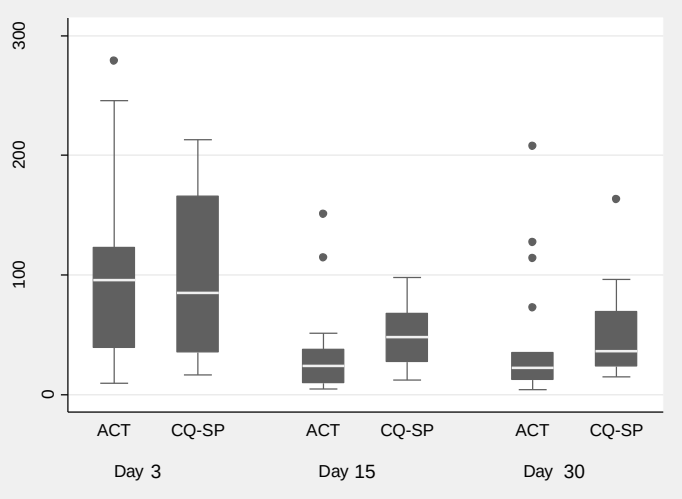
From Day 30 onwards there was a larger Hb response in children who received anti-malaria therapy with CQ-SP compared to ACT. However, the drop in Hb from Day 0 to Day 3 and Day 7 in children who were initially treated with ACT was smaller than the drop in children initially treated with CQ-SP, although the difference was not significant resulting in similar levels of final Hb as indicated in the analysis to determine independent predictors of Day 90 Hb, in which Hb at Day 0 was the strongest predictor.

Figure S2. Percentage of participants with sub-microscopic malaria (qPCR) over time in the placebo group (2007 study) by initial anti-malaria treatment



There was no effect of initial anti-malaria treatment on parasite clearance up until 3 months post-malarial episode.

Figure S3. Box and whisker plot of urinary neopterin in the placebo group (2007 study) by initial anti-malaria treatment



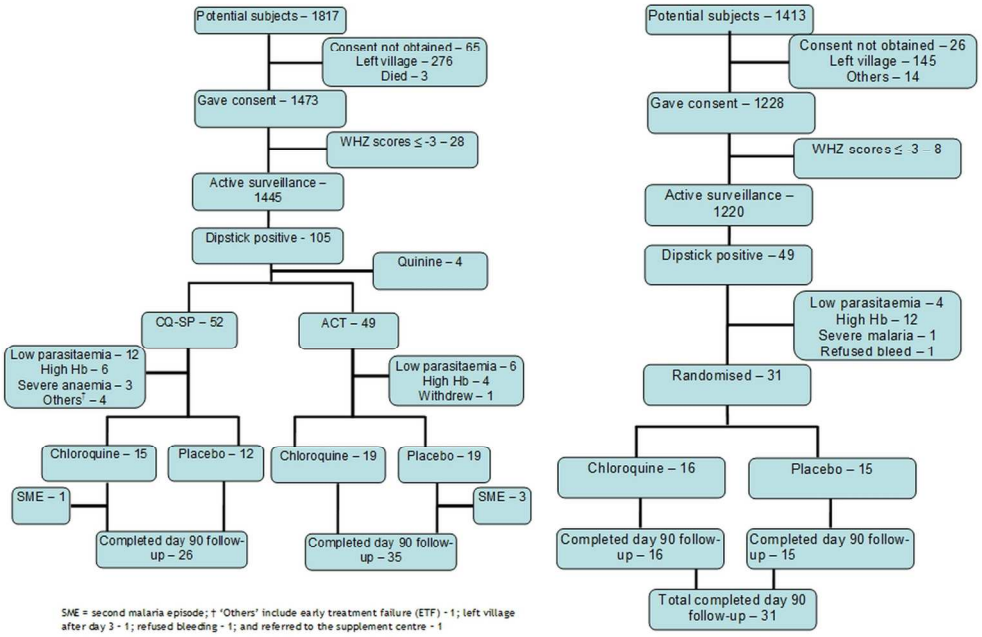
CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2-3
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	6-7
	2b	Specific objectives or hypotheses	7
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	8
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	8
Participants	4a	Eligibility criteria for participants	8-9
	4b	Settings and locations where the data were collected	8
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	**10-11** & 9-10
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	**11**, 9-10, & 12
	6b	Any changes to trial outcomes after the trial commenced, with reasons	N/A
Sample size	7a	How sample size was determined	11-12
	7b	When applicable, explanation of any interim analyses and stopping guidelines	N/A
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	10
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	10
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	10
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	10
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	10

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	10-11
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	12-13
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	12-13
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	14 & Fig1
	13b	For each group, losses and exclusions after randomisation, together with reasons	14
Recruitment	14a	Dates defining the periods of recruitment and follow-up	8 & 4
	14b	Why the trial ended or was stopped	N/A
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	14-15 &Table 3
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	15-18
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	15-18 Tables 3,4a, 4b, Figs 2-6
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	N/A
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	N/A
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	N/A
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	2, 19-21
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	19-21
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	19-21
Other information			
Registration	23	Registration number and name of trial registry	1
Protocol	24	Where the full trial protocol can be accessed, if available	N/A
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	1

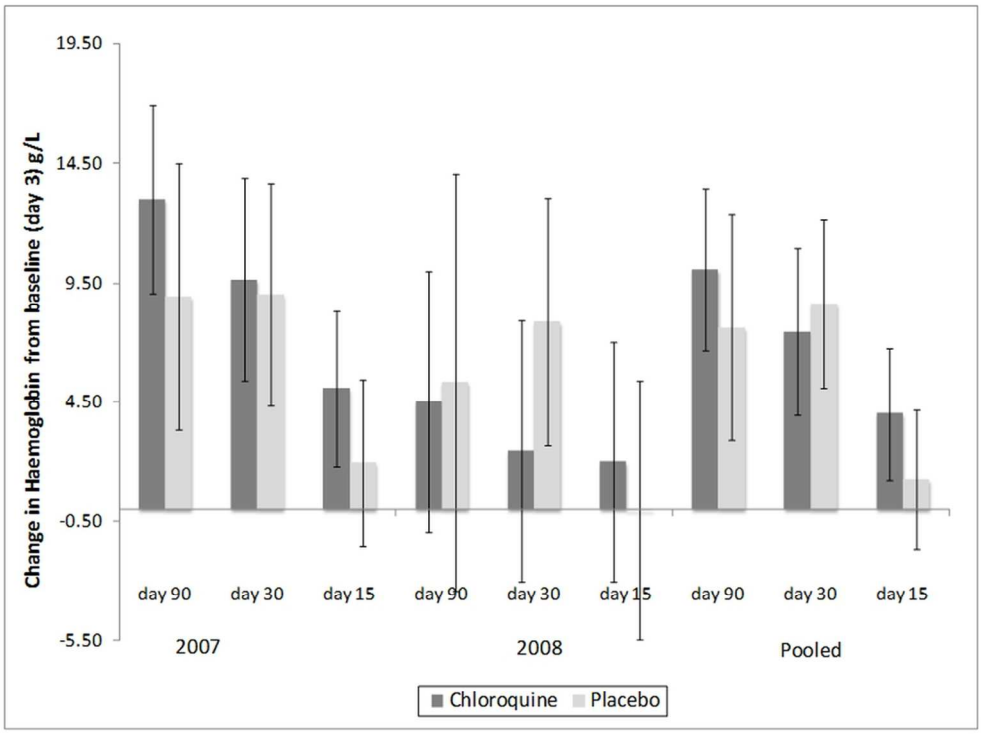
*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

Figure 1 CONSORT Flow Diagram describing participants' movements in 2007 & 2008



130x90mm (300 x 300 DPI)

Figure 2 Mean change in haemoglobin concentration at day 30 and day 90 from baseline at day 3 by treatment group for each year of study and for both years combined.



Error bars represent the 95% confidence intervals

103x90mm (300 x 300 DPI)

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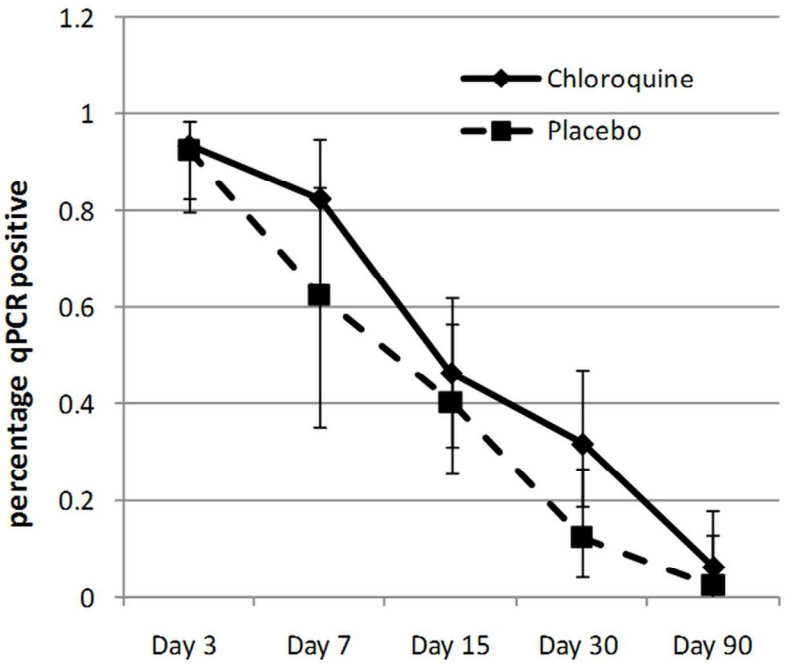

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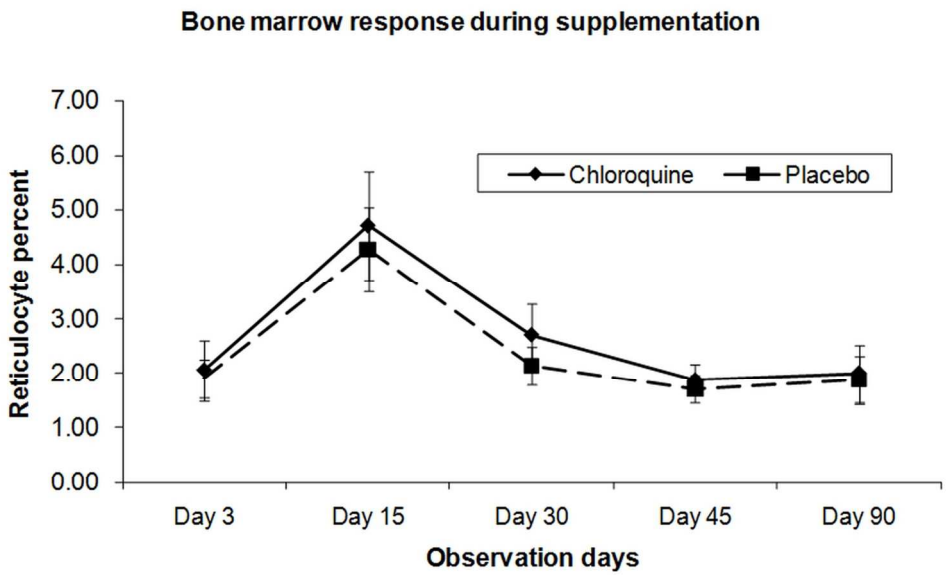
Figure 4. Reticulocyte percentage as an indicator of bone marrow response by treatment group for both years combined.



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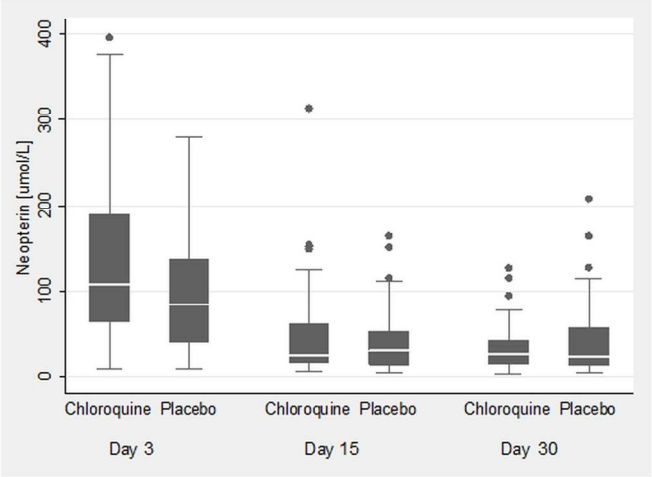

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