THE LANCET Haematology

Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

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APPENDIX MATERIAL

Impact of red blood cell variants on childhood malaria in Mali: a prospective cohort study

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Contents

Appendix 1: Supplementary methods

Clinical procedures

At the clinic in Kenieroba, study clinicians assessed each presenting child using a structured case report to document historical details, vital signs, and physical examination findings, and examining thick and thin Giemsa-stained smears of finger-prick blood samples for malaria parasites. We defined falciparum malaria as axillary temperature >37.5^{o}C (or history of fever within the previous 24 hours) and asexual *P. falciparum* parasitaemia, without other obvious causes of fever. While it is possible that some children had incidental *P. falciparum* infection but another cause of fever, all children improved clinically after receiving only antimalarial treatment (see later). We used World Health Organization severe malaria criteria[1] to define "major-severe" cases as those with cerebral malaria, severe malarial anaemia, respiratory distress, or some combination thereof, and "minor-severe" cases as those with hyperparasitaemia (>100,000 parasites/μL), severe prostration, repetitive vomiting, an inability to tolerate oral therapy, or some combination thereof. Patients lacking any of these severe malaria criteria were diagnosed with mild malaria.

Patients with mild malaria were treated orally with artesunate (4 mg/kg) and amodiaquine (10 mg/kg) each day for 3 days per Mali's national malaria treatment guidelines, and screened for parasites on the 4th day to assess response. Patients classified as either "major-severe" or "minor-severe" were treated intravenously with quinine over 4 hours in 5% or 10% dextrose solution, according to the patient's weight: 20 mg/kg initial dose, then 10 mg/kg every 8 hours until the child could take oral medication, at which time therapy was completed with a 3-day regimen of artesunate and amodiaquine, as for mild malaria.

Quantifying time at risk

Malaria is seasonal in the study villages and lasts from approximately June through December. The limits of the transmission season were defined *a posteriori*. From 2008 through 2011, study clinicians staffed the village clinic in early June and began to assess children with fever. The beginning of the transmission season was defined as the date of the first case of malaria. The end of the transmission season was defined as the date of the last case of malaria, after which no further cases presented for 1 week. These dates were used to initially quantify the number of weeks at risk of malaria and thus child-years of follow-up.

From this basal time at risk, we deducted weeks based on several data. In order to quantify in- and out-migration of study participants (typically to the low endemicity area in and around Bamako), we conducted a census at the conclusion of each transmission season. All participants were queried regarding travel outside the study sites during the transmission season; for participants no longer living in the villages, we queried family members as to their date of departure. These weeks away from the villages were deducted from the child-years of follow-up. Additionally, we deducted 2 weeks of risk for each episode of malaria because monodesethylamodiaquine – the main active metabolite of amodiaquine used to treat all cases – has a long elimination half-life[2] and thus provides a short period of protection from *P. falciparum* reinfection.

The incidence rates calculated with these child-years of follow-up (cyfu) should not be confused with the expected annual rate of malaria incidence in Kenieroba during this period. A child with the typical rate of 1.5 episodes per cyfu that never leaves the village has on average 27.25 weeks of follow up per year (an average of 30.25 weeks during a malaria season minus 3 weeks not at risk because of treatment). Since there are 52.18 weeks in a year, we observe and overall rate of 1.54 episodes/cyfu, and we can assume no incidents during the off-season, the overall crude rate per year in Kenieroba during the study period (2008-2011) is about 1.54*(27.25/52.18)=0.8 episodes per child per full year.

Sample size considerations

Power calculations were done by simulation, assuming a sample size of 1000 and a 5-year study (for a total of 5000 person-years). The simulations used estimates of rates of each of the RBC variants from preliminary data in nearby Kangaba and Kela villages. For the simulations, we used Poisson models including all 5 RBC variants with a sandwich estimator of variance, and (for simplicity) no covariates. We simulated many different levels of incidence rate ratio (IRR), and gave two tables of simulated power based on incidence rates of 400 or 200 *P. falciparum* malaria cases annually. These tables showed that even if we correct for testing 6 effects (G6PD-def is tested twice, once for males and once for females), there is a sufficient power to see reasonably sized effects. For example, with

400 cases annually, we see at least 80% power if the IRR was at or lower than: 0.65 (HbC), 0.7 (G6PD-def males), 0.75 (G6PD-def females), 0.75 (HbS), 0.80 (α-thalassemia), and 0.85 (type O blood group).

Statistical methods

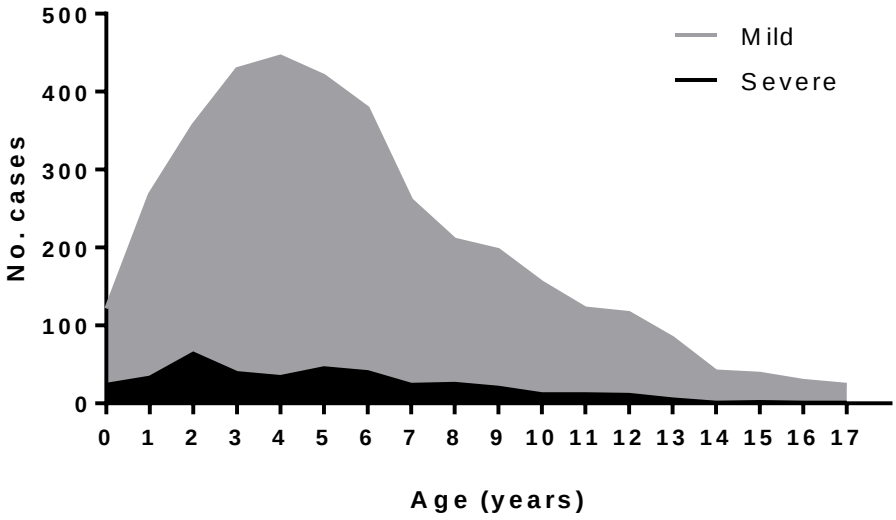
The covariates included in the multivariate models for the quasi-Poisson and GEE parasitemia models were: age (categorical, 1 category per year), ethnicity, β-globin and α-globin variants, ABO blood group, and G6PD A-genotype. The models for incidence (e.g., table 2) also adjusted for year. Because of the way G6PD was entered into the models by sex, there was no need to separately adjust for sex. We do not include many other covariates that affect the outcome (e.g., bednet use, entomological inoculation rate). We assume that these other covariates are independent of the RBC variants, so they would have little confounding effect and do not need to be included in the models since our interest is on the effect of the RBC variants on the outcome. For simplicity and to avoid overfitting due to trying an excessive number of models, we generally assume the effects do not change over levels of the covariates, except where noted (see e.g., Appendices 5 through 8). The quasi-Poisson models allow for extra-Poisson variation using a linear over-dispersion parameter, and were run using the glm function in R (version 3.1.1). Wald tests and confidence intervals were used for IRRs and adjusted IRRs. To test for effects of severe (defined as either "minor-severe" or "major-severe" as above) *vs.* mild malaria, we used score tests from GEE logistic regression models, where the response is whether each malaria episode was severe or not. GEE is needed to account for within-subject correlation. To test for differences within RBC variants or between HbAA and HbAS, the model included an adjustment for age only. The model for the overall β-haemoglobin effect did not converge because there were 0 severe events for HbCC children, so those children were removed and the effect was still significant. For all the GEE models we used exchangeable correlation and tested for effects using score test.

Our primary interest is in 5 RBC variants, represented by 6 hypothesized effects (since we estimate G6PD-deficiency differently for males and females). We make no correction for multiple comparisons for simplicity of presentation, and because each of the 6 hypothesized effects had been studied previously and are intrinsically of interest.

To test for an age-modifying effect to the HbS effect, we considered only HbS and HbAA children and tested for the significance of an interaction term of HbS with age (as a continuous variable) in the multivariate models (quasi-Poisson for IRR and GEE for the parasite density) with covariates as described above, including the main effects for age as categorical. The resulting test assumes a constant change per year of age (i.e., a linear effect on age for the interaction). To estimate or test for percent changes in rates or IRR per year of age, we used two-point estimators of annual percent change, since that estimator does not require constant change assumption[3] and for the rates gives confidence intervals based on transforming exact Poisson confidence intervals.

The GEE models were fit with SAS (version 9.3), the ARR confidence intervals were calculated in Stata/IC (v11), and all other analyses were done in R (version 3.1.1).

Appendix 2: Proportions of mild and severe malaria cases by age.



Severe case definitions are described in **Appendix 1**.

Appendix 3: Characteristics of malaria episodes.

	No. episodes	No. mild (%)	No. severe (%)	p-value
Total	4091	3697 (90.4)	394 (9.6)	
Year				
2008	756	705 (93.3)	51 (6.7)	<0.0001
2009	1174	1073 (91.4)	101 (8.6)	
2010	1111	1022 (92)	89 (8)	
2011	1050	897 (85.4)	153 (14.6)	
Age				
0	153	127 (83)	26 (17)	0.0021
1	300	269 (89.7)	31 (10.3)	
2	421	357 (84.8)	64 (15.2)	
3	478	437 (91.4)	41 (8.6)	
4	476	443 (93.1)	33 (6.9)	
5	469	422 (90)	47 (10)	
6	409	371 (90.7)	38 (9.3)	
7	287	263 (91.6)	24 (8.4)	
8	231	205 (88.7)	26 (11.3)	
9	219	200 (91.3)	19 (8.7)	
10	163	150 (92)	13 (8)	
11	137	126 (92)	11 (8)	
12	123	112 (91.1)	11 (8.9)	
13	89	84 (94.4)	5 (5.6)	
14	41	40 (97.6)	1 (2.4)	
15	40	38 (95)	2 (5)	
16	30	29 (96.7)	1 (3.3)	
17	25	24 (96)	1 (4)	
β-globin variant				
HbAA	3343	3000 (89.7)	343 (10.3)	0.0190
HbAS	408	386 (94.6)	22 (5.4)	
HbAC	321	294 (91.6)	27 (8.4)	
HbCC	4	4 (100)	0 (0)	
HbSC	12	11 (91.7)	1 (8.3)	
HbSS	3	2 (66.7)	1 (33.3)	
α-globin variant				
Normal (αα/αα)	2876	2592 (90.1)	284 (9.9)	0.4795
α^{+} thal ($-\alpha^{3.7}/\alpha\alpha$)	1127	1023 (90.8)	104 (9.2)	
α^{0} thal ($-\alpha^{3.7}/-\alpha^{3.7}$)	88	82 (93.2)	6 (6.8)	
ABO blood group				
A	1266	1138 (89.9)	128 (10.1)	0.9140
B	936	847 (90.5)	89 (9.5)	
AB	327	297 (90.8)	30 (9.2)	
O	1562	1415 (90.6)	147 (9.4)	
G6PD A- genotype				
Boys				
Normal	1837	1666 (90.7)	171 (9.3)	0.9145
A- hemizygotes	197	175 (88.8)	22 (11.2)	
Girls				
Normal	1621	1463 (90.3)	158 (9.7)	
A- heterozygotes	419	378 (90.2)	41 (9.8)	
A- homozygotes	17	15 (88.2)	2 (11.8)	

Severe case definitions are described in **Appendix 1**.

Appendix 4: Modification of HbAS and HbAC effects on malaria risk by α-thalassaemia.

	cyfu	No. events	Incidence rate (events/cyfu)	IRR* (95% CI)	p-value*	IRR** (95% CI)	p-value**
HbAA							
αα/αα	1502.1	2346	1.562	REF	REF	REF	REF
$-\alpha^{3.7}/\alpha\alpha$	546.3	932	1.706	1.07 (0.977,1.171)	0.1448	1.08 (0.987,1.181)	0.0944
$-\alpha^{3.7}/-\alpha^{3.7}$	43.7	65	1.486	1.139 (0.849,1.53)	0.3850	1.151 (0.861,1.538)	0.3437
HbAS							
αα/αα	265.0	280	1.057	0.656 (0.566,0.761)	<0.0001	0.662 (0.572,0.767)	<0.0001
$-\alpha^{3.7}/\alpha\alpha$	109.2	121	1.108	0.687 (0.552,0.854)	0.0008	0.698 (0.563,0.866)	0.0011
$-\alpha^{3.7}/-\alpha^{3.7}$	3.9	7	1.808	1.164 (0.48,2.822)	0.7375	1.133 (0.473,2.715)	0.7796
HbAC							
αα/αα	123.9	231	1.865	1.24 (1.055,1.457)	0.0091	1.235 (1.053,1.448)	0.0095
$-\alpha^{3.7}/\alpha\alpha$	39.8	74	1.858	1.074 (0.815,1.416)	0.6118	1.021 (0.776,1.344)	0.8823
$-\alpha^{3.7}/-\alpha^{3.7}$	4.1	16	3.901	1.565 (0.87,2.818)	0.1352	1.455 (0.81,2.616)	0.2096
Overall epistasis effect* **					0.6322		0.4821

cyfu, child-years of follow-up; REF, reference group; IRR, incidence rate ratio; CI, confidence interval

* Adjusted for age only

** Adjusted for age, year, ethnicity, β-globin and α-globin variants, ABO blood group, and G6PD A- genotype

*** Overall test for interaction of β-globin and α-globin effects using only subjects included in this table (that is, subjects with HbAA, HbAS, or HbAC)

Appendix 5: Modification of HbAS effect on malaria risk by age.

Age	All children			HbAA			HbAS			IRR (95% CI)*	p-value
	cyfu	No. events	Incidence rate	cyfu	No. events	Incidence rate	cyfu	No. events	Incidence rate		
0	185.7	153	0.824	144.1	125	0.868	23.5	8	0.34	0.392 (0.185,0.833)	0.0155
1	174.2	300	1.722	133.6	242	1.812	25.7	28	1.089	0.601 (0.391,0.923)	0.0208
2	174.6	421	2.411	133.8	336	2.512	28.1	46	1.635	0.651 (0.459,0.922)	0.0163
3	172.0	478	2.779	135.0	405	3	27.6	45	1.628	0.543 (0.392,0.751)	0.0003
4	178.1	476	2.673	140.7	383	2.722	27.0	58	2.145	0.788 (0.577,1.077)	0.1356
5	180.7	469	2.595	143.0	397	2.775	25.4	41	1.615	0.582 (0.391,0.866)	0.0080
6	177.0	409	2.311	139.3	333	2.39	24.7	33	1.338	0.56 (0.363,0.863)	0.0091
7	177.1	287	1.621	135.5	236	1.741	25.8	25	0.968	0.556 (0.34,0.908)	0.0196
8	165.5	231	1.396	126.7	177	1.397	24.2	26	1.072	0.768 (0.47,1.255)	0.2927
9	156.5	219	1.399	124.1	184	1.483	22.0	22	1	0.674 (0.366,1.243)	0.2076
10	148.4	163	1.099	118.4	135	1.14	19.9	18	0.906	0.794 (0.43,1.468)	0.4635
11	147.1	137	0.931	118.5	113	0.954	19.0	14	0.736	0.772 (0.4,1.49)	0.4410
12	149.5	123	0.823	122.0	96	0.787	18.9	14	0.739	0.94 (0.487,1.814)	0.8526
13	126.7	89	0.702	103.0	78	0.757	17.1	6	0.351	0.463 (0.168,1.272)	0.1368
14	107.1	41	0.383	86.2	29	0.336	16.5	11	0.667	1.981 (0.856,4.584)	0.1118
15	98.3	40	0.407	78.8	33	0.419	14.8	6	0.404	0.966 (0.3,3.11)	0.9544
16	75.5	30	0.398	60.4	24	0.398	9.1	3	0.328	0.825 (0.168,4.05)	0.8135
17	61.7	25	0.405	49.0	17	0.347	8.5	4	0.473	1.365 (0.358,5.205)	0.6492

cyfu, child-years of follow-up; IR, incidence rate; IRR, incidence rate ratio; CI, confidence interval

* HbAS *vs* HbAA

Appendix 6: Modification of HbAS and HbAC effects on parasite density by α-thalassaemia.

	cyfu	No. events	Median parasite density (IQR)	p-value*	p-value**
HbAA					
αα/αα	1502.1	2346	14800 (4075,30925)	REF	REF
$-\alpha^{3.7}$/αα	546.3	932	16262.5 (4825,32100)	0.6549	0.5228
$-\alpha^{3.7}/-\alpha^{3.7}$	43.7	65	14250 (3275,28950)	0.6841	0.6949
HbAS					
αα/αα	265.0	280	10400 (1400,26400)	0.0030	0.0027
$-\alpha^{3.7}$/αα	109.2	121	12750 (1250,26250)	0.0746	0.0815
$-\alpha^{3.7}/-\alpha^{3.7}$	3.9	7	6750 (550,7050)	0.3567	0.3522
HbAC					
αα/αα	123.9	231	14750 (5550,29000)	0.2265	0.2614
$-\alpha^{3.7}$/αα	39.8	74	11637.5 (1950,23650)	0.3435	0.3308
$-\alpha^{3.7}/-\alpha^{3.7}$	4.1	16	11687.5 (5325,17075)	0.9904	0.6292
Overall epistasis effect* **				0.6787	0.6509

cyfu, child-years of follow-up; IQR, interquartile range; REF, reference group

* Adjusted for age only

** Adjusted for age, ethnicity, ABO blood group, and G6PD A- genotype

*** Overall test for interaction of β-globin and α-globin effects using only subjects included in this table (that is, subjects with HbAA, HbAS, or HbAC)

Appendix 7: Modification of HbAS effect on parasite density by age.

Age	All children			HbAA			HbAS			p-value*	p-value**
	cyfu	No. events	Median parasite density (IQR)	cyfu	No. events	Median parasite density (IQR)	cyfu	No. events	Median parasite density (IQR)		
0	185.7	153	10650 (2875,23100)	144.1	125	11650 (3275,24200)	23.5	8	5000 (675,8850)	0.2782	0.3329
1	174.2	300	18600 (4800,43350)	133.6	242	21150 (7275,47600)	25.7	28	4200 (250,8250)	0.0047	0.0009
2	174.6	421	24300 (9225,51700)	133.8	336	25725 (11550,53200)	28.1	46	12450 (2900,30600)	0.0047	0.0085
3	172.0	478	21375 (9150,43650)	135.0	405	22475 (9825,45325)	27.6	45	14162.5 (1425,26100)	0.0114	0.0114
4	178.1	476	21225 (7950,37675)	140.7	383	21862.5 (9675,38800)	27.0	58	18487.5 (775,32325)	0.0082	0.0093
5	180.7	469	17075 (5775,32625)	143.0	397	17850 (6250,33975)	25.4	41	10637.5 (1250,24150)	0.0434	0.0334
6	177.0	409	14550 (5700,28900)	139.3	333	14350 (5600,29050)	24.7	33	21600 (9750,36650)	0.2062	0.1620
7	177.1	287	13100 (2025,26075)	135.5	236	13250 (2100,25400)	25.8	25	15762.5 (1250,27325)	0.9141	0.7771
8	165.5	231	10650 (1850,22825)	126.7	177	10762.5 (1875,23400)	24.2	26	6875 (1075,23675)	0.4086	0.4077
9	156.5	219	7200 (1525,18750)	124.1	184	6375 (1325,16325)	22.0	22	13550 (5600,24750)	0.0896	0.1292
10	148.4	163	5925 (950,15000)	118.4	135	6837.5 (950,16200)	19.9	18	2400 (750,11175)	0.7483	0.8654
11	147.1	137	6975 (2000,15975)	118.5	113	6900 (2000,13500)	19.0	14	12825 (550,24000)	0.5203	0.7113
12	149.5	123	5475 (425,11525)	122.0	96	5150 (525,10800)	18.9	14	1650 (125,10350)	0.2566	0.2138
13	126.7	89	7000 (700,11700)	103.0	78	5125 (700,11400)	17.1	6	11250 (5275,18000)	0.0525	0.0859
14	107.1	41	2825 (275,8700)	86.2	29	2700 (375,10000)	16.5	11	4087.5 (150,6750)	0.9927	0.6096
15	98.3	40	6625 (825,15750)	78.8	33	6225 (1050,15300)	14.8	6	8287.5 (600,15750)	0.8481	0.2497
16	75.5	30	2250 (525,10050)	60.4	24	2250 (525,9150)	9.1	3	15900 (450,15900)	0.3771	0.5057
17	61.7	25	3100 (350,8600)	49.0	17	3100 (200,7725)	8.5	4	5000 (475,8800)	0.4220	0.7930

cyfu, child-years of follow-up; IQR, interquartile range. Cyfu and number of events in HbAA and HbAS children do not sum to those in all children, owing to additional cyfu and events in HbAC, HbCC, and HbSC children.

* HbAS *vs* HbAA, from univariate model

** HbAS *vs* HbAA, from multivariate analyses that included ethnicity, α-globin genotype, ABO blood group, and G6PD A- genotype

Appendix 8: STROBE statement checklist for cohort studies.

	Item No	Recommendation	Page
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	**1-2**
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	**2**
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	**3**
Objectives	3	State specific objectives, including any pre-specified hypotheses	**3, Appendix 1**
Methods			
Study design	4	Present key elements of study design early in the paper	**3**
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	**3**
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	**3**
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	**N/A**
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	**3-4, Appendix 1**
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	**3-4**
Bias	9	Describe any efforts to address potential sources of bias	**3-4**
Study size	10	Explain how the study size was arrived at	**3**
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	**4**
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	**4**
		(*b*) Describe any methods used to examine subgroups and interactions	**4**
		(*c*) Explain how missing data were addressed	**4**
		(*d*) If applicable, explain how loss to follow-up was addressed	**4**
		(*e*) Describe any sensitivity analyses	**N/A**
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—e.g. numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analyzed	**4**
		(b) Give reasons for non-participation at each stage	**4**
		(c) Consider use of a flow diagram	**4**
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	**4**
		(b) Indicate number of participants with missing data for each variable of interest	**4**
		(c) Summarize follow-up time (e.g., average and total amount)	**4**
Outcome data	15*	Report numbers of outcome events or summary measures over time	**4**
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence interval). Make clear which confounders were adjusted for and why they were included	**4-6**
		(*b*) Report category boundaries when continuous variables were categorized	**4-6**
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	**4-6**
Other analyses	17	Report other analyses done—e.g. analyses of subgroups and interactions, and sensitivity analyses	**4-6**
Discussion			
Key results	18	Summarize key results with reference to study objectives	**6-8**
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	**6-8**
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	**6-8**
Generalizability	21	Discuss the generalizability (external validity) of the study results	**6-8**
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	**9**

Criteria adapted from von Elm *et al.*[4]

Appendix 9: Supplemental references.

1. Severe falciparum malaria. World Health Organization, Communicable Diseases Cluster. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* Apr 2000;94 Suppl 1:S1-90.
2. Krishna S, White NJ. Pharmacokinetics of quinine, chloroquine and amodiaquine. Clinical implications. *Clinical pharmacokinetics.* Apr 1996;30(4):263-299.
3. Fay MP, Tiwari RC, Feuer EJ, Zou Z. Estimating average annual percent change for disease rates without assuming constant change. *Biometrics.* Sep 2006;62(3):847-854.
4. von Elm E, Altman DG, Egger M, Pocock SJ, Gotzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *PLoS medicine.* Oct 16 2007;4(10):e296.