PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Asthma and atopic dermatitis are associated with increased risk of clinical Plasmodium falciparum malaria
AUTHORS	Paul, Richard; Herrant, Magali; Loucoubar, Cheikh; Bassene, Hubert; Goncalves, Bronner; Boufkhed, Sabah; Diene Sarr, Fatoumata; Fontanet, Arnaud; Tall, Adama; Baril, Laurence; Mercereau-Puijalon, Odile; Mecheri, Salaheddine; Sakuntabhai, Anavaj

VERSION 1 - REVIEW

REVIEWER	Nuno Sepulveda, Biostatistician, London School of Hygiene and Tropical Medicine, United Kingdom
REVIEW RETURNED	08-Apr-2013

GENERAL COMMENTS	The manuscript 'Asthma and atopic dermatitis are associated with increased risk of clinical Plasmodium falciparum malaria' suggests a link between allergy and malaria in a cohort of Senegal patients. The paper is easy to ready and the main message is clear. However, there are some aspects that still need clarification in order to fully appreciate the strength and the extent of the claim. **Major comments** The main message of the paper is based on a generalized linear mixed modelling approach applied to the data. Although appropriate in theory, I'm not sure whether this approach provides accurate and robust answers in the present study. Every modelling approach has its own assumptions that must agree with the data. In a setting of a small sample size, specifically in the allergic-related groups, small deviations from model assumptions - for example, a Gaussian distribution is used to analyse the logarithm of maximum parasite density during a malaria episode - may have a strong impact on the corresponding results. In particular, it would be useful to see how the model predictions (and corresponding uncertainties) agree with the empirical cumulative incidence curves shown in Figure 2. Therefore, the authors should do some kind of model checking and validation to support/strengthen their claim.

	The authors assessed age-specific effect of allergy on malaria dividing age into two levels according to the peak of incidence (3.5 years of age, Figure 1). It would be important to check whether the corresponding results remain valid when using alternative age thresholds (e.g., 3.0 and 4.0). It is known that asthma and atopic diseases show gender bias – boys show, in childhood, higher prevalence of asthma than girls and the other way around in adolescence. This putative confounder should also be accounted for in all analyses. In the same line it would be interesting to modify Table 1 in order to include the summary data split by gender. The authors used a pedigree-based analysis because the individuals are genetically related to each other. In this setting, the authors should provide more information about the genetic relatedness among the individuals (how many different families/households/average kinship degree?) and how it was actually calculated. This information is crucial to understand the extent of the claim, specifically when the sample size is rather small. The force of infection was approximated by the ratio between the total number of clinical P. falciparum episodes during the trimester and the total number of person-trimester surveyed. To widen the readability of the paper, it would be interesting to plot the force of infection (and its precision) as function of time and check whether the corresponding curve agrees with what it is known about the malaria dynamics of the study area (internal control of the analysis/data). This point is out of my expertises but I don't understand how none anti-P. falciparum IgE was detectable in the samples. As this is a high malaria transmission area, I was expecting some kind of immunity in, at least, older children during a malaria episode. I would appreciate to read the authors' view on that. **Minor comments** In the Introduction the authors say that some genome wide studies have identified chromosomal regions shared between malaria and asthma, atopic dermatitis, etc. To boost interest of the reader, the authors should provide some interesting examples of those regions. The same applies to the sentence regarding a mouse model of

	atopic disease and in the discussion. With respect to Figure 1, the fitted polynomial trendline is clearly overestimating the incidence curve (R2=0.9855). It would be interesting to have the quadratic and cubic polynomial curves in the same plot. The authors used a pedigree-based genetic relatedness matrix to introduce correlation between individual. How was this matrix calculated/estimated? The association of allergy classes with IgE levels were based on a GLMM with a Poisson distribution. I do not fully understand how one can come up with such probability distribution (used for count data) to describe IgE levels usually considered as a continuous quantitative variable. **Editorial comments** 1. In the abstract it should be clearly stated the total number of individuals included in the analysis. 2. Remove the percentages from the limits of the OR CI in the abstract. 3. In last paragraph of page 9, it reads 'Analysis by allergy category…double the risk of P. falciparum episodes (OR=2.12…)'. It is more correct to say 'Analysis by allergy category… increases the risk of P. falciparum episodes (OR=2.12…)'. The same applies to the following sentence about atopic dermatitis ('tripled' should be replaced by 'increased'). 4. I spotted few typos along the manuscript. See, for example, Figure 2 ('fine lines' and 'indivudals').

REVIEWER	Carlota Dobaño, PhD Associate Research Professor CRESIB I have no competing conflict of interest
REVIEW RETURNED	08-Apr-2013

THE STUDY	Although generally answered YES, some sections lack sufficient details, as specified below, and others need to be revised by experts in the field
GENERAL COMMENTS	The study is conducted in the well-known cohort of Dielmo, Senegal, extensivelly characterised from the malaria point of view over the

	last years, to test the hypothesis that allergy impacts upon clinical Plasmodium falciparum malaria. ABSTRACT - Please specify the sample size of the cohort and age ranges at which the cross-sectional allergy study is conducted INTRODUCTION - Th2 bias in malaria is questionable, proinflamatory and Th1 responses are more often detected. Actually, the Th1 predominance driven by the intracellular parasite may contribute to the significantly lower prevalence of allergies in malaria endemic African populations. Please reconsider this argument as groundwork for the study. METHODS - Please specify number of children included when cohort is described and age range at which the allergy cross-sectional was conducted (not just the max of 15 yr) - Even if previously published, for those not familiarised with the Dielmo studies, please state more explicitly surveillance method to detect and define clinical malaria in this cohort, as this is a key endpoint (passive or active detection, of infection or case, and if active how often, treatment protocol, etc) - Explain rationale for helminth diagnosis - A clinician/pediatrician with expertise in allergy to assess to what extent the adapted ISAAC questionnaire in this population is sufficiently reliable as main source of diagnostic of allergy phenotypes - A statistician/epidemiologist to better assess the suitability of statistical methods RESULTS - Analyses were adjusted by exposure level, how was exposure determined? DISCUSSION - In addition to eluding to the role of cells of the innate immune system in pathogenesis, I find that a more in-depth discussion on putative underlying mechanisms of the adaptive immune system (e.g. Th1 vs Th2) is lacking. OTHER COMMENTS A concern related to the allergy study would be age, if interpreted correctly. In northern countries restricting an asthma diagnosis to children with onset of symptoms before age 2 years would be questionable. The study probably focused on this group to assess asthma before the age peak for malaria. The risk is to capture a large number of early transient wheezers (e.g. because viral infections) and miss a significant proportion of cases with later-onset allergic asthma. However, these phenotypes may be different in the Senegal population. The large range of age at recruitment and relatively small sample size of the cohort are limitations, despite the unique nature and novelty of the longitudinal cohort.

VERSION 1 – AUTHOR RESPONSE

Reviewer 1

1. ABSTRACT

- Please specify the sample size of the cohort and age ranges at which the cross-sectional allergy study is conducted

This has been added

A clinical and immunological allergy cross-sectional survey in a birth cohort of 175 children from 1 month to 14 years of age followed for up to 15 years in a longitudinal open cohort study of malaria in Senegal. Malaria incidence data were available for 143 of these children (aged 4 months to 14 years of age) for up to 15 years.

2. INTRODUCTION

- Th2 bias in malaria is questionable, proinflamatory and Th1 responses are more often detected. Actually, the Th1 predominance driven by the intracellular parasite may contribute to the significantly lower prevalence of allergies in malaria endemic African populations. Please reconsider this argument as groundwork for the study.

This section has been substantially altered as suggested.

It now reads

The World Allergy Organization estimates that 40% of the world's population is concerned by allergic diseases.1 In developing countries where Plasmodium falciparum malaria is endemic, prevalence of allergy is significantly lower, but is on the increase.2 Orientation of the immune response towards a Th1 profile is crucial for immunity to intracellular pathogens,3 whereas orientation towards a Th2 profile drives immunity to extracellular pathogens and antigens resulting in class switching giving rise to IgE-producing B cells.4 An important role of the Th1/Th2 balance in the development of clinical malaria following infection by P. falciparum has been suggested by numerous studies.5-7 It has been suggested that the Th2 bias induced by P. falciparum may exacerbate allergy.8 Likewise, an atopic state may generate a tendency to develop a Th2 type immune response to P. falciparum. However, the interplay between infectious agents and allergy is unclear. On the one hand, for example, severe respiratory syncytial virus infection in infants increased the risk of allergic rhinoconjunctivitis and allergic asthma.9,10 On the other hand, measles,11 hepatitis A12 and tuberculosis13 seemingly reduce atopy. Although, an atopic condition can increase incidence of disease, such as the case for the skin commensal Staphylococcus aureus in patients with atopic dermatitis,14 an atopic tendency per se does not generally lead to increased illness from infectious agents.

3. METHODS

- Please specify number of children included when cohort is described and age range at which the allergy cross-sectional was conducted (not just the max of 15 yr)

We have added the following

175 children aged from 1 month to 14 years old who were born during the malaria research program.

4. - Even if previously published, for those not familiarised with the Dielmo studies, please state more explicitly surveillance method to detect and define clinical malaria in this cohort, as this is a key endpoint (passive or active detection, of infection or case, and if active how often, treatment protocol, etc)

Added the following

In brief, between 1990 and 2008, a longitudinal study involving the inhabitants of the village of Dielmo, Senegal, was carried out to identify all episodes of fever. The study design included daily medical surveillance with systematic blood testing of individuals with fever and examination of 200 oil-immersion fields on a thick blood film for malaria parasites (about 0.5 µL of blood). Each individual

was given a unique identification code and details of family ties, occupation, and precise place of residence were recorded on detailed maps of each household with the location of each bedroom. All households were visited daily, absenteeism recorded, and the presence of fever or other symptoms assessed. We systematically recorded body temperature at home three times a week (every second day) in children younger than 5 years, and in older children and adults in cases of suspected fever or fever-related symptoms. In cases of fever or other symptoms, blood testing was done at the dispensary by finger prick, and we provided detailed medical examination and specific treatment. Parasitologically confirmed clinical malaria episodes were treated according to national guidelines. From 1990 to 2008, four different drug regimens were implemented: Quinine from 1990 to 1994, Chloroquine from 1995 to 2003, Fansidar (sulfadoxine-pyrimethamine) from 2004 to mid-2006 and Artemisinin-based combination therapy (ACT; Amodiaquine- sulfadoxine-pyrimethamine) from mid-2006 to 2008.
Parasite positivity was established as follows. Thick blood films were prepared and stained by 3% Giemsa stain. Blood films were examined under an oil immersion objective at x1000 magnification by the trained laboratory technicians and 200 thick film fields were examined to count the number of asexual and gametocyte parasite stages. Asexual parasite densities (per µL) were calculated by establishing the ratio of parasites to white blood cells and then multiplying the parasite count by 8,000, the average white blood cell count per µL of blood.

5. - Explain rationale for helminth diagnosis
Added the following

Helminthic infections are common in this region and are known to modify the clinical course and outcome of both allergic diseases and malaria.29,30

6. - A clinician/pediatrician with expertise in allergy to assess to what extent the adapted ISAAC questionnaire in this population is sufficiently reliable as main source of diagnostic of allergy phenotypes

We developed the ISAAC protocol and the clinical allergy examination with the aid of the leading Senegalese paediatric allergologist and a dermatologist at Institut Pasteur clinic.
We have added this in the acknowledgements.
We are indebted to Michel Thiakane, a pediatric allergologist, head of the Pediatric Service, Principal hospital, Dakar, Senegal and Marie-Thérèse Guinnepain, dermatologist, Institut Pasteur clinic, Paris, France for aid in elaborating the ISAAC questionnaire and advising on clinical diagnosis of allergy in Senegal.

7. - A statistician/epidemiologist to better assess the suitability of statistical methods
Several of the co-authors are statisticians and/or epidemiologists (Loucoubar, Fontanet, Baril, Tall), some of whom give courses in statistics and/or epidemiological statistics in Public Health. Dr. Fontanet, for example, is head of the Epidemiology unit at Institut Pasteur and currently Dr. Baril is a chief epidemiologist for GSK. Perhaps the titles appended to the authors (e.g. MD or scientist) are a little imprecise.

RESULTS
8. - Analyses were adjusted by exposure level, how was exposure determined?

This was in the methods. We have added exposure level after force of infection.
We calculated the quarterly incidence rate of clinical P. falciparum episodes in children below the age of 15 years as the ratio of the total number of clinical P. falciparum episodes during the trimester divided by the total number of person-trimesters surveyed. Incidence rate is expressed as cases per 100 person-trimesters. This rate was used in the analysis to approximate the force of infection

(exposure level) within the targeted population at the time of a given clinical P. falciparum episode.

DISCUSSION

9. - In addition to eluding to the role of cells of the innate immune system in pathogenesis, I find that a more in-depth discussion on putative underlying mechanisms of the adaptive immune system (e.g. Th1 vs Th2) is lacking.

We have addressed this and added the following

Although the immune effectors of clinical immunity are still poorly defined, there is strong evidence that acquired anti-parasite immunity is IgG-dependent38 and cytophilic immunoglobulins (IgG1 & IgG3), which are capable of eliminating the parasites by opsonisation and/or by Antibody Dependent Cellular Immunity play an important role in premunition.35 The higher parasite density during symptomatic episodes observed in the asthma group suggests impaired development of acquired immunity. Impaired acquisition of immunity to malaria in children with asthma or atopic dermatitis may stem from their imbalanced Th1/Th2 response. Indeed, an atopic state may generate a tendency to develop a Th2 type immune response to P. falciparum. Dendritic cells that are oriented to a Th2 phenotype are more susceptible to orient the acquired immune response towards a Th2 profile.39 Orientation of the immune response towards a Th2 profile by asthma or atopic dermatitis would result in a poor Th1 response (and hence development of protective IgG immunoglobulins), considered to be the dominant arm of the immune response enabling resistance to infectious disease in children.40

10. OTHER COMMENTS

A concern related to the allergy study would be age, if interpreted correctly. In northern countries restricting an asthma diagnosis to children with onset of symptoms before age 2 years would be questionable. The study probably focused on this group to assess asthma before the age peak for malaria. The risk is to capture a large number of early transient wheezers (e.g. because viral infections) and miss a significant proportion of cases with later-onset allergic asthma. However, these phenotypes may be different in the Senegal population. The large range of age at recruitment and relatively small sample size of the cohort are limitations, despite the unique nature and novelty of the longitudinal cohort.

We agree on this, but only 15 individuals of the 143 for whom full malaria and allergy data were available were under 2 years of age during the allergy study. The remaining 128 individuals had allergy diagnosed when they were over two years of age (and up to 15 years) but for whom malaria data were available since birth. This information is tabulated in Supplementary Table 1. We also add in the text (in strengths and weaknesses section).

In addition, although allergy diagnosis for children under 2 years of age is not considered reliable, there were only 15 individuals under 2 at the time of the allergy study of the 143 for whom malaria and allergy data are available.

Reviewer 2 (next page)

Reviewer 2

1. Model checking/validation

We have added the model predictions for the cumulative incidence of clinical cases in the supplementary material for comparison with Figure 2 and added reference to this Figure S2 in the text.

Figure S2. Cumulative incidence of clinical cases according to allergy class predicted by the statistical

model.

To check the goodness of fit of the model for log parasite density, as classically done, we examine the distribution of the residuals (Figure S3, referenced in the text).

Figure S3. Graphical control model for parasite density
These figures provide a graphical checking of model goodness of fit. Figure A is the scatter plot of the natural logarithm of the observed parasite density and is compared to Figure B, which is the scatter plot of the natural logarithm of the predicted parasite density by the model; on both figures A and B the y-axes give the values for the log of the parasite density. Figure C shows the distribution of the residuals with the predicted values and Figure D is the histogram of the residuals; both figures C and D show the residuals normally distributed around zero.

2. Age-specific effect (3.5 years) – verify using alternative thresholds.

An interesting point.
We have re-run the analyses using age-thresholds of 1.5, 2.5, 3.5, 4.5 and 5.5 years of age. The results remain the same. We have added the following in the text (methods and results) and will add the complete analysis output data in supplementary material (Table S3).

In the methods section

The age threshold was varied from 1.5 years to 5.5 years of age and the data re-analysed to assess at which age there was the strongest effect.

In the results section

Analysis using different age thresholds (from 1.5 to 5.5 years of age) revealed similar OR for thresholds of 2.5, 3.5 and 4.5 years of age. The maximum OR for increased malaria occurred in children older than 4.5 years of age and with atopy or atopic dermatitis, whereas for the asthma group it occurred in children after 3.5 years of age (Supplementary Table).

3. Asthma gender bias. Include gender as a confounder in analysis and in Table 1.
Gender was actually in the original analysis and had no significant effect. We have added this in the results. We have also modified Table 1 to give the gender specific details.

4. Pedigree based analysis – give more information on genetic relatedness.

We have added the following in the main body of the text and the methods in the supplementary material.
Methods:

The family structure (pedigree) was available after a demographic census performed for every volunteer at his adhesion in the project. A verbal interview of mothers or key representatives of the household was used to obtain information on genetic relationships between studied individuals, their children, their parents, and to identify genetic links among the population. The total pedigree

comprised 828 individuals, including absent or dead relatives, composed of ten independent families that can be sub-divided into 206 nuclear families (father – mother couples with at least one child) with an average of 3.6 children each. Genetically related nuclear families occur because of multiple marriages and marriages among related individuals. Previous typing with microsatellites has enabled the construction of a pedigree based on Identity-by-Descent using MERLIN.18,26 The mean coefficient of inbreeding is 0.0008. Newborns since this original genetic analysis were added to the family of the parents in question. The 143 children, with both allergy and malaria data, belonged to 61 nuclear families and comprised 30 singletons, 102 siblings and 11 half-sibs (yielding 55 half-sib pairs). The mean genetic relatedness (by pedigree) of the 143 children is 0.0114 (range: 0.0013 to 0.022).

Supplementary material

Pedigree-based genetic relatedness

The Genetic covariance between two individuals can be computed using the pedigree information. For individuals A and B, a given pair in a pedigree, the genetic covariance is computed as r(A,B) = 2×coancestry(A,B) where the coancestry between A and B is calculated referring to the method presented by Falconer and Mackay in 1996 (Falconer and Mackay 1996): coancestry(A,B) = $\Sigma p(1/2)n(p)\times(1 + I\text{Common Ancestor})$ where p is the number of paths in the pedigree linking A and B, n(p) the number of individuals (including A and B) for each path p and IX is the inbreeding coefficient of X also equal to the coancestry between the two parents of X, IX is set to 0 if X is a founder.

Illustration: Consider, as an example, the pedigree below containing 18 individuals named {A, B, …, R} for the calculation of genetic covariance's.

Pedigree structure.

The genetic relatedness between individuals N and O is equal to 0.266. This value is calculated as followed:
The number of paths linking N and O from the pedigree structure above is p = 2.
As illustrated below:
• Path 1 contains n(1) = 3 individuals {N, K, O} with K as the common ancestor. Inbreeding coefficient of K, IK , is the coancestry between the two parents of K (F and G) and is null because F and G are not genetically linked.
• Path 2 contains n(2) = 7 individuals {N, K, F, B, H, L, O} with B as the common ancestor. Inbreeding coefficient of B, IB , is null because B is a founder.
Therefore, genetic relatedness between individuals N and O is:
$= 2\times(0.5\ n(1)\times(1+IK) + 0.5n(2)\times(1+ IB))$
$= 2\times(0.5\ 3\times(1+0) + 0.5\ 7\times(1+0)) = 0.266$

Path 1 linking N and O. Path 2 linking N and O.

Defining an equivalent model design where individual effects are independent using the genetic relatedness matrix:

Let us rename $Y^* = l(\mu)$. Y^* can be consider as a linearization of the phenotype through the link function l. The expected mean of Y^* and the variance of Y^* are:

(i) $E(Y^*) = E(X\beta + Z\gamma + \varepsilon)$
$= E(X\beta) + E(Z\gamma) + E(\varepsilon) = X \times E(\beta) + Z \times E(\gamma) + E(\varepsilon)$
$= X\beta$ (asymptomatically).

(ii) $Var(Y^*) = Var(X\beta + Z\gamma + \varepsilon)$
$= Var(Z\gamma + \varepsilon)$ (as $X\beta$ is the fixed part, thus has variance equal to 0)
$= Var(Z\gamma) + Var(\varepsilon)$ (as γ and ε are independent)
$= Z \times Var(\gamma) \times ZT + Var(\varepsilon)$ (ZT is the transpose of Z)
$= Z(A\sigma g2)ZT + I\sigma r2$
$= ZAZT\sigma g2 + I\sigma r2$

If individuals were independent, i.e. $A = IN$, variance of Y^* could be expressed as $ZZT\sigma g2 + I\sigma r2$. However, using linear algebra theory by the method “Cholesky decomposition of a matrix”, we can show that there is an equivalent expression of the variance of Y^* corresponding to the modeling of data from independent individuals, having γ^* as an equivalent vector of random effects and Z^* an equivalent design matrix relating γ^* to Y^* so that:
$Var(Y^*) = Z^*(I\sigma g2)Z^*T + I\sigma r2$. $I\sigma g2$ is then the covariance matrix of the equivalent independent random individual effects γ^*.

Theorem: Cholesky decomposition of a matrix
If A is a symmetric positive-definite matrix, there is a triangular matrix L so that A can be written as $A = LLT$. L can be seen as the “square root” of the matrix A.
Note that the genetic relatedness matrix A computed using the pedigree information (Falconer and Mackay 1996) is a positive-definite matrix, unless identical twins are in the pedigree in which case it would be positive semi-definite.

Equivalent model with independent random effects: We set $A = LLT$ then:
$Var(Y^*) = Z(A\sigma g2)ZT + I\sigma r2$
$= Z(LLT\sigma g2)ZT + I\sigma r2$
$= ZLLTZT\sigma g2 + I\sigma r2$
$= (ZL)(ZL)T\sigma g2 + I\sigma r2$
$= (Z^*)(Z^*)T\sigma g2 + I\sigma r2$ (where we set $Z^* = ZL$)
Then, if we define $\gamma^* = L^{-1}\gamma$, we can rewrite the model as:
$Y^* = X\beta + Z^*\gamma^* + \varepsilon$ (because $Z\gamma = Z(LL^{-1})\gamma = (ZL)(L^{-1}\gamma) = Z^*\gamma^*$),
and the γi^* are independent, in other terms $Var(\gamma^*) = I\sigma g2$, as demonstrated below:
We assumed that $\gamma \sim N(0, A\sigma g2)$. Then $\gamma^* = L^{-1}\gamma$ is also distributed as a multivariate Normal with mean $E(\gamma^*) = L^{-1}E(\gamma) = L^{-1} \times 0 = 0$ and variance:
$Var(\gamma^*) = (L^{-1}) \times Var(\gamma) \times (L^{-1})T$
$= (L^{-1}) \times A\sigma g2 \times (L^{-1})T = (L^{-1})LLT(L^{-1})T\sigma g2$
$= (L^{-1}L)(L^{-1}L)T\sigma g2$
$= I\sigma g2$

The random effects are now independent and then the classical mixed model assuming independence between levels (here individuals) is applied, and the estimate of fixed effects obtained are fine, i.e. corrected for genetic relationships.
References
Falconer DS, Mackay TFC (1996) Introduction to Quantitative Genetics. 4th Edn. London: Longman.

5. Plot force of infection as a function of time and compare with what is known about malaria dynamics in area.
We have added the force of infection graph in the supplementary material. It is difficult to know how to compare this with what is known about the malaria in the site, because this plot describes the malaria

dynamics in the site. The number of episodes concerns only the under 15 year olds, but these make up over 95% of the cases.

6. IgE absence?
In fact, we have noticed this before in another study in an area of highly seasonal transmission. After the malaria season, the IgE titres drop, most likely because they are bound to effector cells. Thus, it is not that there is no IgE but no circulating IgE. In addition, in the context of responses to the other reviewer, we have added the following.

There was no evidence of anti-parasite IgE in this cohort of children. We previously showed that circulating anti-parasite IgE titres were strongly positively correlated with anti-mosquito saliva IgE and became undetectable following malaria exposure, potentially being bound to effector cells.31

Minor
1. Include examples of genetic region loci shared between malaria and allergy.
We have added the following in the introduction.
Chromosomal region 5q31 that has been repeatedly shown to be associated with control of parasite density and contains a cluster of cytokines, among which IL12B has been previously associated with psoriasis.19 The other regions 13q13-q22, 5p15-p13 and 12q21-q23, contain genes involved in innate immunity, notably the interleukin 7 receptor, and several involved in tumour necrosis factor synthesis [C1q and tumour necrosis factor related protein 3 (C1QTNF3)] and a gene involved in the complement system (C9).

This region contains several candidate genes that have effects on T-cell function.21

2. Figure 1 Plot 2nd and third order polynomials as well.
This we have done

3. Pedigree-matrix calculation
Given. See response to point 4.

4. IgE stats
Error on our part. As in our previous article, IgE data were box-cox transformed as they were not normally distributed (Shapiro Wilks test $P<0.001$) and then analysed with a normal distribution. This has been corrected.

Editorial comments

1. Abstract – number of individuals
Added
2. Remove %s
Removed
3. Replace doubled/tripled by increased
Replaced
4. Typos corrected

VERSION 2 – REVIEW

REVIEWER	Nuno Sepulveda, Biostatistician, London School of Hygiene and Tropical Medicine, United Kingdom
REVIEW RETURNED	29-May-2013

THE STUDY	The statistical analysis of parasitemia still needs to be refined by the authors because model assumptions do not seem to agree with data as explained in my comments to the authors.
RESULTS & CONCLUSIONS	Although the results seem credible and plausible, the statistical analysis of parasite density may not be statistically robust as explained in my comments to the authors. Therefore I recommend another round of revision so the authors can sort that issue out.
GENERAL COMMENTS	General comments: The manuscript entitled 'Asthma and atopic dermatitis are associated with increased risk of clinical Plasmodium falciparum malaria' has significantly improved since the original submission and I congratulate the authors for their great effort in providing more information on how they conducted the data analysis. This version is by far more interesting to read than the previous one. The results seem overall robust but there is still a single point that needs further clarification. Although the overall conclusion seems credible and plausible, the model fitted to parasite density data shows lack of statistical validity. To be more precise, it is said in Figure S3 that the residuals are normally distributed and centred in zero – one of the model assumptions (what was the Box-Cox parameter used?). However, the histogram of the (standardised) residuals (Figure S3D) suggests a skewed-to-the-left distribution. The authors should clarify/discuss this issue or fit an alternative statistical model. In the latter option I recommend the use of a mixed model approach based on an extreme value distribution (since the parasite density was defined as the maximum parasitemia during a malaria episode). Editorial comments: 1. In the Abstract (Design) it should be said that the results

	were also adjusted for gender. 2. On top of page 11, 'there were 2,065 treated P. falciparum clinical episodes (median 11, range 0-47)'. Are these last values per individual? That is, is the median number of malaria episodes per individual equal to 11? 3. In page 12, third paragraph, replace 'yielded the same pattern' by 'yielded similar qualitative conclusions'. 4. In page 13 (Meaning of the Study), 'we show here that children with clinically defined asthma or atopic dermatitis had a two to three-fold increase…'. Rigorously speaking it is better said 'we show here that children with clinically defined asthma or atopic dermatitis have an increased risk…'. 5. On top of page 14, replace 'A previous study' by 'A previous study in Ethiopia (East Africa)..' 6. Check the lines in Figure 1. They do not seem to be right labelled.

REVIEWER	Carlota Dobaño, PhD Associate Research Professor CRESIB, Catalonia, Spain I have no competing interests.
REVIEW RETURNED	16-May-2013

GENERAL COMMENTS	Most of my comments have been well addressed, there only remain a couple of concerns: INTRODUCTION - Th2 bias in malaria is questionable, proinflamatory and Th1 responses are more often detected. Actually, the Th1 predominance driven by the intracellular parasite may contribute to the significantly lower prevalence of allergies in malaria endemic African populations. Please reconsider this argument as groundwork for the study. Although the authors did include a new paragraph about this topic, the sentence "It has been suggested that the Th2 bias induced by P. falciparum may exacerbate allergy (ref 8)" remains in the article. There is some inconsistency between this statement and the sentence above "Orientation of the immune response towards a Th1 profile is crucial for immunity to intracellular pathogens". Even though there is a reference given to a Commentary in AJTMH, to my understanding the larger body of evidence shows a Th1 bias in malaria infection, and not a Th2 bias (Pf is an intracellular parasite); Th2 biass is suggested by elevated IgE in some instances by the commentary reference provided, and more to do with infection

	chronicity (children would not be initially under chronic infection, this would come later in life). Therefore I suggest to rethink again the way this Th1/Th2 balance is formulated and presented in the introduction. - There is still some confusion about the last comment raised in the original review. Based on your definition, the child would have severe asthma (which accounts for the large majority of asthma cases) if <the child had "wheezing or whistling in the chest before the age of two years" and "more than three times" or severe enough to "limit his/her speech">. So, if this is understood correctly, somebody who was seen say at age 10 years could be still classified as asthmatic even if (s)he had sympotms ONLY in the first two years of life, or is this a wrong assumption? In other words, were asthma symptoms required to be current at the time of the survey? Little is known about phenotypic manifestation of asthma/allergy in Senegal to really confirm whether this is a real concern, but the way it has been addressed is not completely convincing.

VERSION 2 – AUTHOR RESPONSE

Reviewer 1

Introduction

Question 1: TH2 bias in malaria questionable. Proposed use of malaria reducing atopy as basis for study. Sentence with reference (8) unconvincing.

Response:

Whilst it might be tempting to use "the infectious agent protecting from allergy" thesis as a basis to the study, this would seem strange in the light of colleagues' genetics findings (overlapping regions linked to clinical malaria and atopy) that are discussed in the following paragraph.

However, in an attempt to reduce the emphasis on Th2 and malaria, we have altered this paragraph. This notably meant removing the sentence and reference 8 as suggested.

- We have added "T helper type 2 (Th2) cells, their related cytokines, IgE, eosinophils and mast cells play a major role in allergic inflammation."
- We have removed important from "An important role of the Th1/th2 balance….."
- We have replaced the sentence "It has been suggested that the Th2 bias induced by *P. falciparum* may exacerbate allergy.[8]"

With

"Whilst it is recognised that acquired anti-parasite immunity is IgG dependent,[8] parasite-specific IgE also impact upon the clinical outcome of infection. For example, higher IgE but not IgG levels have been observed in patients with cerebral malaria than those with uncomplicated P. falciparum infection.[9] The role of IgE, however, remains unclear. [10]"

Old ref 38 is new reference 8 and new refs 9 & 10. (Old ref 8 deleted)

- Deleted “Likewise, an atopic state may generate a tendency to develop a Th2 type immune response to *P. falciparum*.”

Question 2: There is still some confusion about the last comment raised in the original review. Based on your definition, the child would have severe asthma (which accounts for the large majority of asthma cases) if <the child had “wheezing or whistling in the chest before the age of two years” and “more than three times” or severe enough to “limit his/her speech”>. So, if this is understood correctly, somebody who was seen say at age 10 years could be still classified as asthmatic even if (s)he had symptoms ONLY in the first two years of life, or is this a wrong assumption? In other words, were asthma symptoms required to be current at the time of the survey? Little is known about phenotypic manifestation of asthma/allergy in Senegal to really confirm whether this is a real concern, but the way it has been addressed is not completely convincing.

Response:

Most children who have asthma develop their first symptoms before 5 years of age (50-80%). Moreover, certain clinical signs of disease increase the likelihood that a given infant with wheeze has (or will be diagnosed with) atopic asthma. One of them is the frequency of wheezy episodes before the age of two years, associated or not with respiratory infection. According to the internationally recognised ISAAC questionnaire, asthma symptoms do not need to be current at the time of the survey to assess the prevalence of asthma in children. In addition, we deliberately chose to prioritize the specificity of the allergic status assessment by giving weight to questions concerning asthma symptoms highly scored according to the respective medical diagnosis of paediatricians and paediatric allergologists established in previous studies carried out in subtropical countries.

Reviewer 2

Editorial comments

1. Results adjusted for gender in Abstract (Design) – added

2. Yes 2,065 treated P. falciparum clinical episodes (per individual: median 11, range 0-47) – added

3. Replaced as suggested

4. Replaced as suggested

5. Replaced as suggested

6. The problem has seemingly arisen in the conversion of word doc to pdf. Fixed, thanks.

Statistical comments on analyses of parasite density

We have revised and re-analysed as follows and will add this as a supplementary document and make reference to this in the text in the results section concerning analysis of parasite density.

“As the log-transformed data were left skewed, we additionally analysed using box-cox transformation and probit normalization of the data. The results were qualitatively the same (Supplementary text and Figures S4-S8).”

The model we fitted on the parasite density ("*pf_density*") has used as outcome variable the natural logarithm of *pf_density* (equivalent to a Box-Cox for which the parameter is null). As shown on Figure S04 the distribution of log(*pf_density*) is not perfectly normal, it is left-skewed.

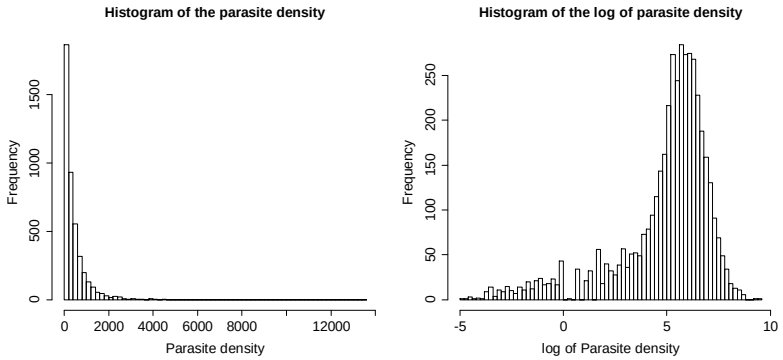

Figure S04. Histogram of *pf_density* and log(*pf_density*)

Taking into account the reviewer comments, we add here the case for a Box-Cox transformation of the parasite density where the parameter is λ = 0.3, this parameter value was obtained as optimal using the R- function named "boxcox" from the "MASS" library. Then the Box-Cox transformation of the parasite density is $y = (pf\_density^{0.3} - 1)/0.3$ having the distribution shown on Figure S05 below.

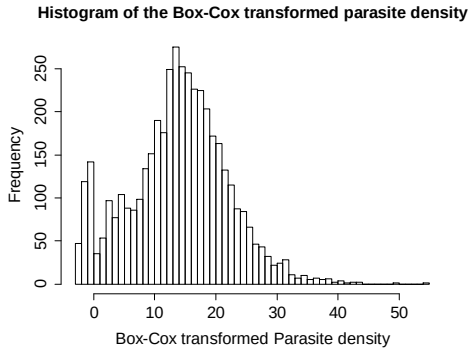

Figure S05. Histogram of the Box-Cox transformation of pf_density using a λ parameter of 0.3

With this Box-Cox transformed parasitemia as outcome variable, our results are maintained as summarized below. Note that this distribution is not "perfectly" normal. However, the corresponding graphical control of the model adequation presented on Figure S06 below shows residuals more close to the normal distribution than those for log(*pf_density*) as outcome.

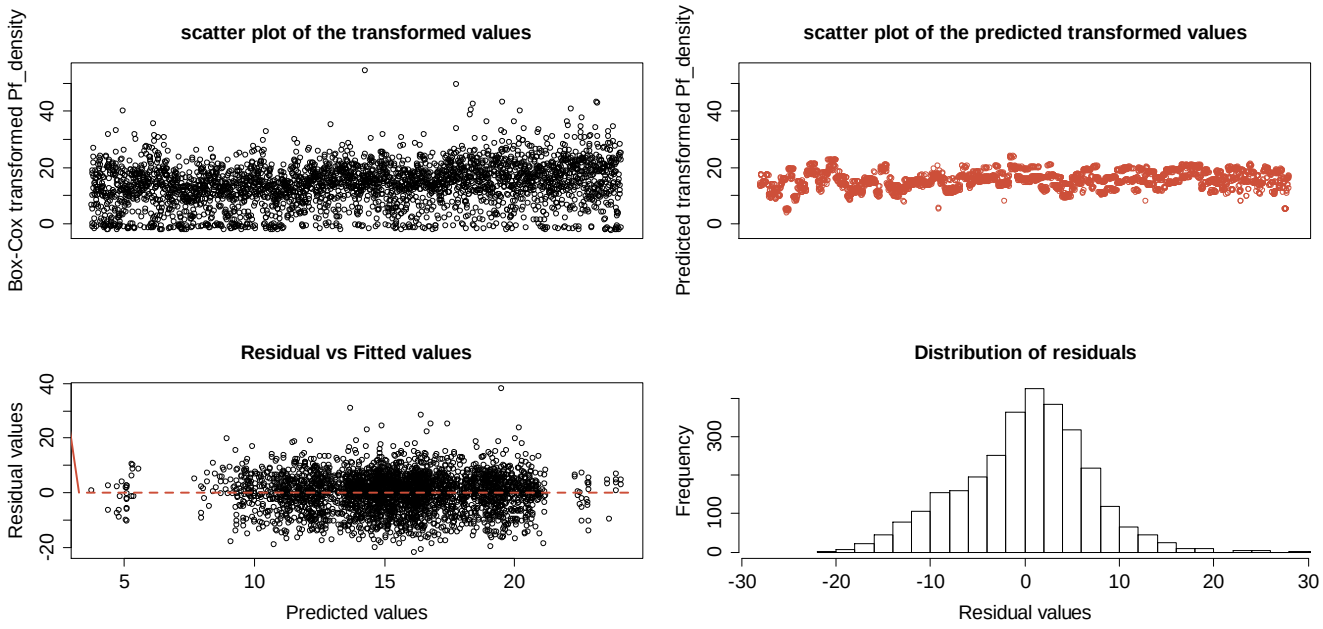

Figure S06. Graphical control of the model adequation for *y* = Box-Cox(*pf_density*, λ = 0.3)

Also, we agree with the remark of the reviewer saying that histogram of residuals suggests a left-skewed distribution when log(*pf_density*) was used as outcome. The reviewer recommended a mixed model approach based on an extreme value distribution. However, the method we used incorporating pedigree information was developed through an R-package known as "pedigreemm" that allows just for a limited number of distribution laws, which do not include extreme value distributions like the Gumbel or Weibull distributions.

However, we tried the Probit normalization on the log(*pf_density*) to readjust its quantiles to those from a standard normal, and subsequently used the derived standard normal transformation of the log(*pf_density*) as outcome (see Figure S07 below, the three graphs presented in the first row of the graphs panel concern the log(*pf_density*) before Probit normalization and the three in the second row are for after Probit normalization. We can see on the histogram in blue color a good normal distribution of the *y* variable.

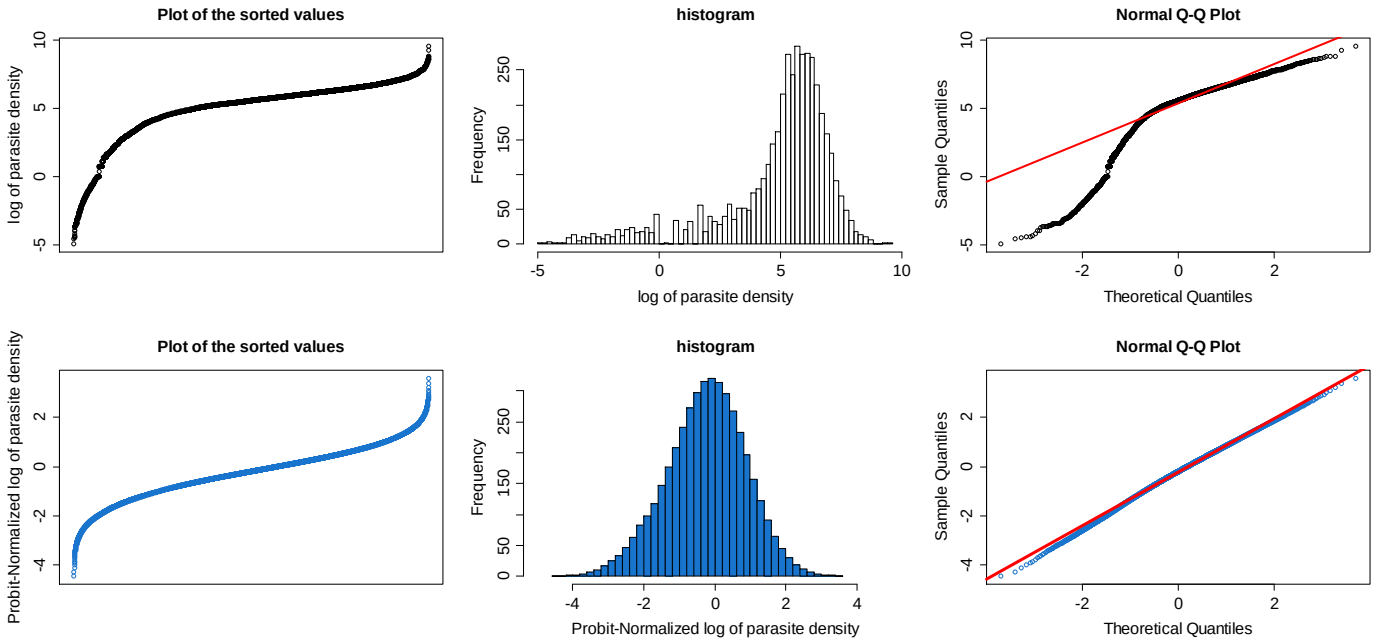

Figure S07. Probit normalization of the log(*pf_density*)

The results we obtained after this Probit normalization of the log(*pf_density*) confirmed the same findings as summarized below. Also, the corresponding graphical control of the model adequation presented on Figure S08 below, shows a good normal distribution of residuals from this model.

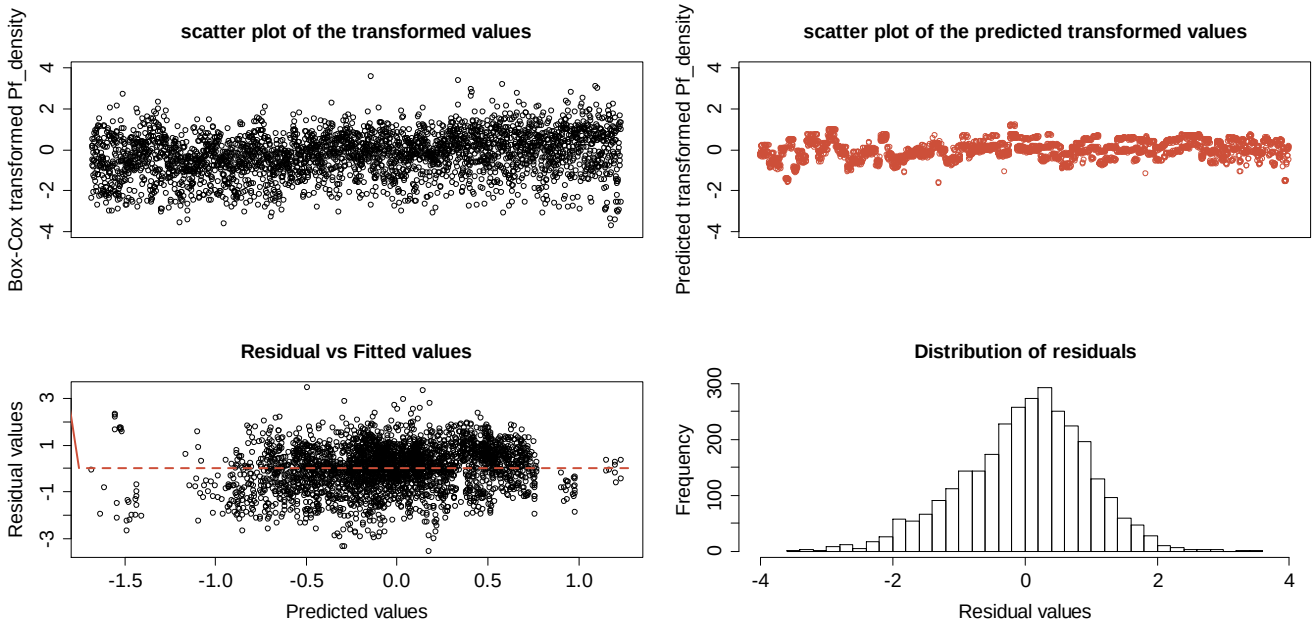

Figure S08. Graphical control of the model adequation after Probit normalization of the log(*pf_density*)

Results of new statistical analyses

*** logPf after normalization by Box-Cox Transformation**

* agegroup0: < 3.5y

* agegroup1: >= 3.5y

* ATOPY (all together)

	beta	se	z	Pvalue
(Intercept)	13.60124135	0.714338179	19.040339	0.000000e+00
agegroup1	-3.37771380	0.333032920	-10.142282	0.000000e+00
incidence_per_trim	0.01542103	0.003645885	4.229707	2.339956e-05
agegroup0:atopy	1.17191285	0.928015888	1.262816	2.066555e-01
agegroup1:atopy	2.85234256	0.920799095	3.097682	1.950407e-03

* ASTHMA

	beta	se	z	Pvalue
(Intercept)	13.69170488	0.709419174	19.2998799	0.000000e+00
agegroup1	-3.20406525	0.318171186	-10.0702559	0.000000e+00
incidence_per_trim	0.01519843	0.003651335	4.1624298	3.148789e-05
agegroup0:asthma	0.96613275	1.122321445	0.8608343	3.893293e-01
agegroup1:asthma	2.30175490	1.069803300	2.1515683	3.143137e-02

* DERMATITIS

	beta	se	z	Pvalue
(Intercept)	13.61569783	0.70752410	19.2441470	0.000000e+00
agegroup1	-3.16640481	0.30811703	-10.2766302	0.000000e+00
incidence_per_trim	0.01605434	0.00366192	4.3841326	1.164488e-05

agegroup0:dermatitis	1.09757825	1.22406154	0.8966692	3.698955e-01
agegroup1:dermatitis	4.04445306	1.38680193	2.9163884	3.541094e-03

* RHINITIS

	beta	se	z	Pvalue
(Intercept)	13.71958091	0.720933690	19.0302951	0.000000e+00
agegroup1	-3.11948595	0.319703167	-9.7574446	0.000000e+00
incidence_per_trim	0.01529699	0.003660078	4.1794158	2.922589e-05
agegroup0:rhinitis	0.67336585	1.099403424	0.6124829	5.402183e-01
agegroup1:rhinitis	1.50750888	1.116269849	1.3504879	1.768595e-01

*** logPf after normalization by Probit Transformation (agegroup0: < 3.5y, agegroup1: >= 3.5y)**

* ATOPY (all together)

	beta	se	z	Pvalue
(Intercept)	-0.322269575	0.1013066957	-3.181128	1.467028e-03
agegroup1	-0.451825156	0.0463733984	-9.743197	0.000000e+00
incidence_per_trim	0.002335765	0.0005077129	4.600562	4.213517e-06
agegroup0:atopy	0.175613789	0.1318337400	1.332085	1.828322e-01
agegroup1:atopy	0.394919286	0.1309863260	3.014966	2.570083e-03

* ASTHMA

	beta	se	z	Pvalue
(Intercept)	-0.30921378	0.1004172939	-3.0792881	2.074959e-03
agegroup1	-0.43058811	0.0442927045	-9.7214229	0.000000e+00
incidence_per_trim	0.00230148	0.0005083086	4.5277208	5.962328e-06
agegroup0:asthma	0.15488975	0.1590019225	0.9741376	3.299882e-01
agegroup1:asthma	0.33678313	0.1518862735	2.2173375	2.660004e-02

* DERMATITIS

	beta	se	z	Pvalue
(Intercept)	-0.315751017	0.1002420046	-3.1498873	1.633335e-03
agegroup1	-0.427439497	0.0428898322	-9.9659867	0.000000e+00
incidence_per_trim	0.002423605	0.0005097226	4.754753	4 1.986888e-06
agegroup0:dermatitis	0.138918766	0.1735226271	0.8005801	4.233748e-01
agegroup1:dermatitis	0.575782719	0.1958780334	2.9394961	3.287464e-03

* RHINITIS	beta	se	z	Pvalue
(Intercept)	-0.304393783	0.1022711044	-2.9763420	2.917094e-03
agegroup1	-0.416126237	0.0445085427	-9.3493566	0.000000e+00
incidence_per_trim	0.002320589	0.0005096242	4.5535290	5.275338e-06
agegroup0:rhinitis	0.101347270	0.1561114479	0.6491982	5.162103e-01
agegroup1:rhinitis	0.193876631	0.1586201197	1.2222701	2.216055e-01