Author’s response to reviews

Title: Modulation of innate immune responses at birth by prenatal malaria exposure and association with malaria risk during the first year of life

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A formatted authors' response letter has been included as a supplementary file.
Response to Reviewer’s comments

We thank the Reviewer#2 and the BMC Medicine editorial member for carefully evaluating our revised manuscript and for their constructive criticism. Please see below our point-by-point response to each point raised.

Member of the editorial board:

As we were only able to receive two reports out of three, we sought advice from one of our Editorial Board Members, who provided a number of comments which we believe will help improve your revised manuscript. In particular, we encourage you to clarify the study design, and the clinical implications of your results:

- I found it difficult to understand how many women in each of the four malaria exposure strata had received treatment and how many doses. There was insufficient information about how the clusters were equally represented across the substudy (and 4 malaria exposures subgroups).

RESPONSE: We have reorganized the Table 1 (page 20) into columns based on the main groupings used throughout the manuscript (non-exposed, exposed No PM, past PM, chronic PM, acute PM), which, indeed, is much more informative including treatment received by mothers during pregnancy. This was also suggested by the reviewer#2.

In addition, we added more details regarding the study design in the Methods section (lines 169-177, page 5), highlighting the fact that the stimulation assays of cord blood samples from the mother-child pairs included in the present study were performed blinded to the placenta histology as the placenta biopsies were analysed later on. Consequently, there was no attempt to have equal representation of the clusters across the sub-study (and the 4 PME subgroups) as the PME categorization was carried out afterwards upon the TLR-mediated stimulation assays. However, as we have mentioned in the limitation paragraph (Lines 611-634, pages 12-13), the low numbers in the acute PM group is a reflection of the prevalence of PM categories in the main COSMIC trial as most of malaria infections in the placenta were past or chronic PM (95.5%).

- In addition, the following should be taken into account:

  1) Small numbers in some of the strata, the reviewers suggested merging 2 groups, I couldn't find this information.

RESPONSE: We had addressed this question in the first review round, as follows: “The reason why we analysed these two groups separately was that in previous studies, the impact on immune
response was different for these two groups. Therefore, given that only 7 infants were born from mothers with acute PM, we have analysed the effect of these two categories separately, which gives the opportunity to observe (i) the effect of chronic PM in a non-negligible group (n=38) of individuals and (iii) the trend in infants born to mothers with acute PM”.

The analysis merging chronic and acute PM cases could have been done but we believe that having both groups is actually one of the values of the present study. Even if the number of acute PM cases is particularly low, we think the stratified analysis has a solid biological rationale for the following reasons:

- There is literature evidence that chronic and acute placental infection may elicit different birth outcomes and different immune responses. In fact, chronic but not acute PM has been associated with intrauterine growth retardation, prematurity and LBW (Menéndez et al., 2000 in J Infect Dis). Regarding differences in immune responses, chronic PM has been associated with either higher production of pro- (TNF) and anti-inflammatory (IL-10) cytokines (Boudova et al., 2016 in Open Forum Infect Dis), expansion of malaria-specific FoxP3+ CD4+ Treg cells (Flanagan et al, 2010 in Eur J Immunol), or maturation of myeloid and plasmacytoid dendritic cells in cord blood (Fievet et al., 2009 in Malar J).

- Although it is not known for placental infections, chronic infections induce immunoregulatory mechanisms with an expansion of exhausted cell phenotypes that are not induced in acute infections (Mattatal et al., 2015 in Blood; Hashimoto et al., 2018 in Annual Rev Med). In the case of malaria infections, it has been shown that chronic exposure to malaria is associated with either inhibitory and activation markers on atypical memory B cells and marginal zone-like B cells in pregnant women (Ubillos et al., 2017 in Front Immunol) or phenotypic evidence of B and T cell exhaustion in children (Illingworth et al., 2013 in J Immunol). From experimental infections using mice, it has also been shown that chronic malaria infection is associated with PD-1 dependent exhaustion of CD8+ T cells with hampered ability to control the infection (Horne-Debets et al., 2013 in Cell reports).

Therefore, we opted to sacrifice some statistical power for acute infection and gain specificity of infection groups. Moreover, our results show that acute and chronic may have different cytokine profile (some examples: crude concentrations of IFN-γ, IL-4, MIG, MCP-1 tend to be higher in acute than chronic PM, Supplementary Figure S3).

2) Seasonality of malaria in relation to the timing of pregnancy and subsequent neonatal exposure, something also raised by the reviewers

RESPONSE: We agree that seasonality is a very important factor to consider in our study. This is particularly important in Nanoro where we have described a marked seasonal distribution of malaria infection and disease, with the high transmission period (i.e. July to December)
accounting for 89% and 77% of symptomatic and asymptomatic infections, respectively (see Natama et al., 2018 in Malar J). In addition, we showed how the risk of malaria during the first year of life fluctuates depending on the birth season, suggesting that birth season represents an adequate indicator of the seasonality of malaria in the study area (see also Natama et al., 2018 in J Infect Dis). Furthermore, birth season also indicates timing of pregnancy in relation with the seasonality of malaria. Consequently, although birth season was not significantly associated with time to first clinical episode (Figure 5, P=0.84), we took it into consideration in the Cox proportional hazard analyses through an interaction term between birth season and timing of the clinical malaria occurrence. This is explained in the Results section (Lines 369-372). We had mentioned that in our first response to reviewer’s comments as follows:

“Actually, the study was conducted in a rural area with similar malaria transmission intensity in the health district. The major characteristic of this study area is that malaria transmission is highly seasonal (Natama et al, Mal. J. 2018, 17:163). Therefore to account for difference in the risk of infections between infants, birth season was included in the multivariable models using an interaction term with the timing of clinical malaria. Moreover, PME, which influences level of maternal antibodies, was also included in the multivariate models to control for the effect of in-utero exposure to malaria parasites and/or antigens (Lines 390-399)”.

It is worth noting that birth season was also included in the list of potential confounding factors that influence levels of cytokines in cord blood (Lines 334-339, pages 8-9). Therefore, the timing of pregnancy in relation to malaria seasonality was also controlled for variation in cytokine levels in cord blood.

3) Nulliparous vs multiparous woman -- who have different levels of risk- needs to be highlighted, as it's another variable to account for

RESPONSE: We agree with the member of the editorial board that gravidity is a variable of great importance and we took that into consideration in our study. As we do not have nulliparous women among the study participants, gravidity was categorized in three levels (i.e. primigravid, secundigravid and multigravid women). Then gravidity was used as a covariate when analyzing both factors that influence cytokine levels and factors that influence the risk of malaria during the first year of life. As shown in additional file 3, gravidity is one of the factors that significantly contribute to influence cytokine levels. The other factors taken into account include birth season, sex of the newborn, ethnicity and LBW. We have now clarified in the results section (334-336, pages 8-9) the factors found to be associated to cytokine levels and that were controlled for in subsequent models.

Regarding the role of gravidity and risk of malaria during the first year of life, we have also assessed the association between gravidity and time-to-first malaria exposure. As you can see in the figure below, there was no evidence that gravidity influence the risk of malaria during the
first 12 months of life among the study participants. Consequently, gravidity was not included as covariate in Cox proportional multivariable analyses. We are now mentioning in the result section (Lines 361-365, page 9) the list of potential confounding factors analyzed (i.e. gravidity, PME, LBW, birth season, newborn sex, newborn ethnicity and ITN usage by mothers) among which, PME and LBW were the two factors significantly associated with the risk of malaria during the first year of life.

4) Questions about priming of innate immunity

RESPONSE: Actually, we had previously explained to reviewers (and provided references) and in the manuscript that pathogen exposure can lead to increased innate responses (sensitization), something known as trained innate immunity. We have evoked trained immunity as potential explanation of the observed heightened responsiveness among infants prenatally exposed to malaria, an explanation that reviewer #2 finds plausible. However, we cannot discard potential differences in the cell composition, as we also discuss in the manuscript.

Following reviewer’s suggestions, we have now modified the corresponding paragraph to clearly and more directly state both things (Discussion section, Paragraph 2, Lines 416-420, page 10) and (paragraph 3, lines 422-426 page 10).

- The paper delivers a very complex result with inconsistencies between the exposures ie only result that was significant was past malaria and no malaria exposure. The reviewers too wondered about this.

RESPONSE: We agree with the editor about the complexity of this type of studies, also considering the large amount of data generated. Nonetheless, we believe that the study findings deliver key points with understandable messages. Following the (first) reviewer’s comments, we had modified the abstract and the first paragraph of the discussion to clearly state the main study outcomes. Indeed, our study indicates that differences in infants’ susceptibility to malaria could arise from different types of PME, through their different effects on fetal innate immune responses. Consequently, in addition to the difference in susceptibility to malaria between exposed vs non-exposed infants (extensively reported in the literature), there could be an additional layer of heterogeneity in susceptibility to malaria within the exposed group. As shown in the manuscript (paragraph 2, Lines 407-414, page 10), our findings are consistent with results from several studies showing differences in outcomes and immune responses using different types of PME (compared with our groups) (Prahl et al., 2016 in Malar J; Brustoski et al., 2005 in J Immunol; Breitling et al., 2006 in Infect Immunity; Gbédandé et al., 2013 in Infect Immunity)

In consequence we do not think that there are inconsistencies between the exposure groups since our hypothesis was that there might be differential modulation of the innate immune responses
across PME groups. Past PM represents infections that were resolved some time ago and it is reasonable to expect a differential impact on the placenta/fetus as compared to women with active placental infection at delivery (acute and chronic PM groups). Therefore, we believe the fact that past PM has a more profound effect on fetal immune system followed by chronic PM is perfectly possible, although we acknowledge a potential lack of power in the case of acute PM (Paragraph 6, Lines 611-634, page 12-13).

Our findings are supported by previous studies, which are cited in the manuscript (Paragraph 3, lines 422-429, Page 10). The presence of malaria pigments in the placenta has been associated with expansion of malaria-specific FOXP3(+) Treg and more generalized FOXP3(+) CD4(+) Treg in chronic and past PM, alongside increased Th1 pro-inflammatory responses (INF-γ, TNF-γ and IL10) in resolved PM infection only (Flanagan et al, 2010 in Eur J Immunol). Furthermore, the presence of malaria pigment in the placenta may induce the maturation of DCs, which are important innate immune effector cells (Fievet et al., 2009 in Malar J).

- The clinical risk of malaria (measured by passive surveillance) is not currently written up well, but it is very interesting.

I suspect owing to the design (passive rather than active) may have missed quite a number of cases. Overall the results were not sufficiently explained to provide a robust interpretation.

We agree that active case detection allows to detect more cases than passive case detection. Nonetheless, our study has some specific characteristics as it combined a passive case detection of clinical cases and an active case detection of asymptomatic infection with 3-months intervals. The design of birth cohort study and the follow-up procedures have been extensively described elsewhere (Natama et al, 2018 in Malar J). The characterization of malaria burden in infants in the study area was one of the objectives of our overall project. As described in the Malaria Journal paper, mothers were sensitized to visit the closest peripheral health center at any time their offspring was sick, regardless of symptoms. Through the permanent presence of the field workers in the villages to perform the cross-sectional surveys at 3, 6, 9 and 12 months for each child, mothers were continuously encouraged to visit the health centers for any health issue.

A total of 59% of infants experienced at least one clinical episode during the first year of life. When taking into account the subgroup of infants analyzed in the present immunological study (N=313), 60.4% of them developed a clinical episode. As an indicator of early health seeking behavior, only a few infants developed severe malaria (1.5%) (11 out of 717 clinical episodes recorded among the overall cohort). This was significantly lower than the reported number of SM cases in the health district during the same period (4.2% between 2014-2016).

We have now added in the Results section (Lines 359-361, Page 9) that malaria burden among the overall cohort has been described elsewhere and added data on the incidence of clinical
malaria and the median survival time observed in the subgroup of infants included in the present study. Furthermore, we have extended the last paragraph of the results section (Lines 373-391, pages 9-10) to better highlight the clinical implications of our findings.

- Please add a list of abbreviations

RESPONSE: We have added a list of abbreviations in the manuscript (Lines 643-657, Page 13).

Reviewer #2:

In this revised manuscript, Natama and colleagues address many of the issues raised in the previous review. This reviewer appreciates details included re: parent COSMIC trial and revised figure 1 as well as the revised abstract. I have some suggestions that might increase interpretability of this revised manuscript.

1) Table 1: Would consider reorganizing this table into columns based on the main groupings used throughout the manuscript (non-exposed, exposed No PM, past PM, chronic PM, acute PM). Would be much more informative.

RESPONSE: We thank the reviewer #2 for this suggestion and have modified Table 1 accordingly.

2) Discussion remains too long and rather unwieldy at present. Would try to shorten. Some suggestions below:

- Discussion paragraph 1: Unfortunately, the 2nd -4th sentences do not make sense as written (appears to potentially have been a copy/paste error with one of the limitations sentences with run-on sentences as a result)

"Overall, we found that PME has a profound effect on the fetal immune Second, in this study, the measurement of white blood cells population… thus influence TLR-mediated innate immune responses. system and that the …"
RESPONSE: We apologize for that error. The right sentences have been inserted as it was in the revised manuscript with track changes (Discussion section, Paragraph 1, Lines 395-405, page 10).

- Discussion paragraph 2: Line 388: "However other approaches, regarding the categorization of PME and/or the stimulation assays, have been previously used to…” Unclear what this clause (regarding the categorization of PME and/or the stimulation assays) means. Suggest you remove it.

RESPONSE: This suggestion has been taken into consideration and we have removed that sentence (Discussion section, Paragraph 2, Lines 407-409, page 10).

- Discussion paragraph 3: Agree that "trained" innate immunity could explain the heightened responsivity observed in this study among PM-exposed cord blood samples - the paragraph should state that more directly. However, the main counter to this hypothesis is within the last clause of the paragraph - that it could also be due to altered cellular frequencies in cord blood (and/or influence of other immune cells at present) rather than reprogramming of innate cells.

RESPONSE: We have modified the paragraph 3 accordingly and combined it with paragraph 2 (Discussion section, Paragraph 2, Lines 416-420, page 10).

- Discussion paragraph 4: This paragraph could be combined with the prior paragraph. First three sentences describing categories of PM redundant and unnecessary. Starting at line 420 (It has been previously shown…) - could be moved up to prior paragraph and condensed.

RESPONSE: This paragraph (now paragraph 3) has been modified according to these suggestions (Discussion section, Lines 422-429).

- Line 424, Line 434 - "probably" should be re-written as "possibly."

RESPONSE: We are now using “possibly” instead of “probably” in line 426 and line 498.

- Discussion paragraph 5, 6 read too long. Could remove repetition of results in sentence 2 of paragraph 5 (line 439) and sentence 2-3 from paragraph 6 (455-457).

RESPONSE: These suggestions have been taken into consideration by removing the corresponding sentences (Paragraph 4, Lines 501-512, page 11)
- Line 470: "consisting on" - unclear what this means. Perhaps: "Altogether, our findings are consistent with the hypothesis that PME results in down-regulation of cytokine production that can affect all important functional classes of cytokines…"

RESPONSE: This sentence has been modified accordingly (Lines 541-528, page 11).

- Line 474: "...then also more prone to pathogenesis upon infection." - would remove or state that this is speculative as no data was presented to support this hypothesis.

RESPONSE: We have removed the sentence from the discussion (Lines 541-528, page 11).

- Line 522: "Overall, these results demonstrate that PME has a clear impact on malaria risk." As these are associations would temper this statement and change the word "demonstrate" to "suggest."

RESPONSE: We are now using “suggest” instead of “demonstrate” in that sentence to temper our statement (paragraph 5, Lines 605-609, page 12).