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Letter to the Editor

Asymptomatic *Plasmodium falciparum* infection evades triggering a host transcriptomic response

Dear Editor,

As stated in recent submissions to this Journal, symptomatic infection with *Plasmodium* parasites elicits dynamic and complex host responses that cause changes in haematological, immunological and biochemical indices, with prognostic and diagnostic importance.[1,2] However, asymptomatic *Plasmodium falciparum* infections greatly outnumber symptomatic infections in malaria endemic settings, where they sustain parasite transmission, and present a challenge for malaria elimination efforts.[3] A high prevalence of asymptomatic parasitaemia also dilutes the specificity of malaria diagnosis in acute febrile illness, especially when diagnostic tests for other causes are scarce, resulting in overestimation of the malaria burden and failure to treat other life-threatening infections.[4] Protection from symptomatic disease (clinical immunity) is acquired following repeated malaria infections in high transmission settings.[5] The mechanisms that permit this asymptomatic state are poorly characterised, although exposure-dependent parasite-specific immunoregulatory responses have been postulated.[5] Better understanding of the asymptomatic state is important to develop strategies to detect, treat, and prevent these infections, and to distinguish incidental asymptomatic parasitaemia from symptomatic malaria.

To characterise host responses enabling the asymptomatic state in *P. falciparum*-infection, we recruited children (6–12 years) from Obom, a high malaria transmission community in Ghana.[6] We performed transcriptomic analysis of blood [7] from 44 children: 12 with symptomatic *P. falciparum* infection, 25 with asymptomatic infection, and 7 healthy uninfected children. A detailed summary of materials and methods is provided in Supplementary File 1.

The three groups did not differ from each other in terms of age or sex (Supplementary File 2, Supplementary Table S1). To remove confounding from variation in cellular composition of blood, we initially adjusted for the proportions of CD4 + T cells, CD8 + T cells, B cells, NK cells, γδ T cells, neutrophils, dendritic cells and monocytes, quantified by flow cytometry in 37 of these subjects.[6] 735 genes were differentially expressed between symptomatic (n = 9) and uninfected subjects (n = 7) (Fig. 1A; Supplementary File 1; Supplementary File 2, Supplementary File 3), consistent with previous findings that acute malaria elicits a strong transcriptional response.[8] Contrary to our expectations, no genes were significantly differentially expressed between the larger group of asymptomatically infected children (n = 21) and the healthy uninfected children (n = 7) (Fig. 1B).

Since parasite load is a major determinant of the transcriptional host response in malaria,[8,9] we stratified the asymptomatically infected children into those with microscopically detectable (n = 9) and sub-microscopic (n = 12) parasitaemia, and repeated comparisons with the healthy uninfected children (Fig. 1C, D). Neither analysis revealed any differentially expressed genes (Supplementary File 3).

We repeated a similar analysis on an independent dataset of Gabonese children,[10] adjusting for blood leucocyte mixture. Only one gene (*COX7B*, Cytochrome C Oxidase Subunit 7B) reached borderline significance in differential expression between asymptomatic (n = 5 pooled samples) and uninfected (n = 5 pooled samples) subjects (Supplementary Fig. 1A; Supplementary File 4). We then repeated the analyses of the Ghanaian and Gabonese children without adjustment for cell mixture. Only 1 gene (*SNRPD2P1*: Small Nuclear Ribonucleoprotein D2 Pseudogene 1) passed the nominal significance threshold for differential expression between asymptomatically infected (n = 25) and uninfected (n = 7) Ghanaian subjects (Supplementary Fig. 2). No genes were identified as significantly differentially expressed in the Gabonese subjects (Supplementary Fig. 1B). The lack of reproducibility of these single genes (*COX7B* and *SNRPD2P1*) suggests they are most likely false discoveries.

To exclude heterogeneity within the asymptomatic group as an explanation for the lack of differentially expressed genes, we performed differential expression analysis on 5000 iteratively sampled subsets of the Ghanaian children. In the symptomatic-uninfected comparison, 1021, 2146 and 2814 genes passed the significance threshold in greater than 99%, 90% and 80% of iterations, respectively (Supplementary File 5). No genes in the asymptomatic-uninfected comparison passed the significance threshold in more than 1% of iterations. Principal component analysis and K-means clustering corroborated the similarity of the blood transcriptome between asymptomatic and uninfected children, distinct from the symptomatic children (Supplementary Fig. 3). Raw read counts and metadata of the Ghanaian subjects are included as Supplementary Files 6 and 7 respectively.

To enhance the statistical power of comparisons, we employed two further analysis strategies: weighted gene correlation network analysis (WGCNA),[11] and BloodGen3Module analysis.[12] WGCNA reduces dimensionality (and therefore the false discovery rate penalty for multiple comparisons) by constructing groups of highly co-expressed genes (modules) within a dataset, which can be used as units of analysis.[9,11] WGCNA on the data from the Ghanaian children generated 86 modules when gene expression counts were adjusted for leucocyte mixture, and 47 modules without this adjustment. No modules were significantly differentially expressed between asymptomatically infected and healthy uninfected Ghanaian children in either analysis (Supplementary File 8).

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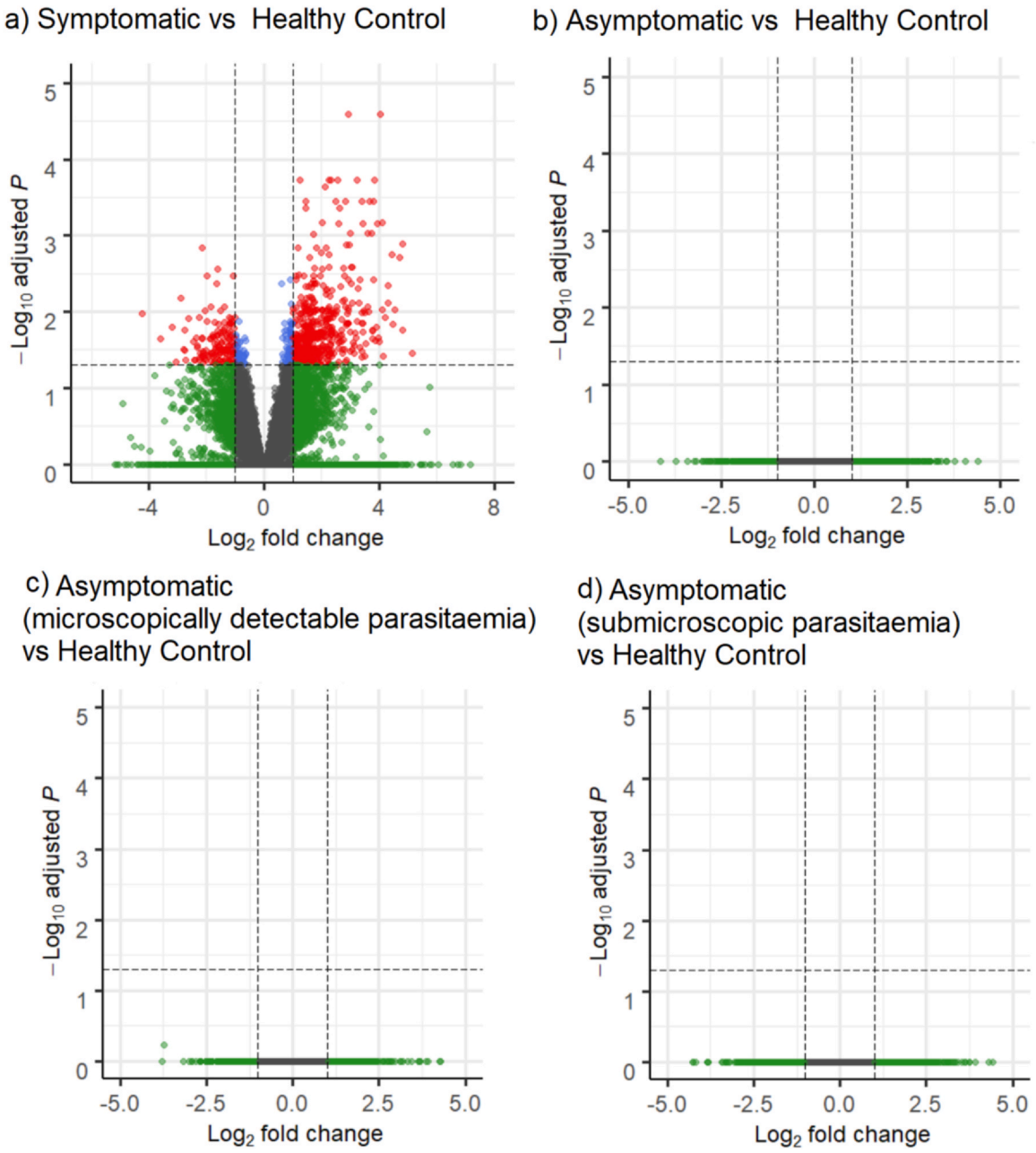

Fig. 1. Volcano plots showing differential gene expression between groups of Ghanaian children. The horizontal dotted lines on each plot show the threshold of a Benjamini-Hochberg false-discovery rate corrected p-value < 0.05 (-log10 value of 1.30). Genes on or above this line are significantly differentially expressed (red, blue). The vertical dotted lines represent log2(fold change) thresholds of −1 and 1 respectively (red, green). Genes considered significantly differentially expressed with absolute log2(fold change) > 1 are coloured red. a) Results of the differential expression between symptomatic (n = 9) and healthy uninfected (n = 7) children. Genes with a log2(fold change) greater than 0 have higher expression in the symptomatic group. Genes with a log2(fold change) less than 0 have lower expression in the symptomatic group. b) Results of the differential expression between asymptomatic (n = 21) and uninfected healthy control (n = 7) children. c) Results of the differential expression between asymptomatic children with microscopically detectable parasitaemia (n = 9) and healthy uninfected children (n = 7). d) Results of the differential expression between asymptomatic children with sub-microscopic parasitaemia (n = 12) and healthy uninfected children (n = 7).

BloodGen3Module, which uses a fixed repertoire of functionally annotated gene sets characterising different immunological and physiological states, was used without adjustment for leucocyte mixture.[12] 8132 genes that passed filtering thresholds in our dataset were members of modules, 72% fewer genes than in the preceding gene level analysis. None of these reached gene-level significance for differential expression between asymptomatically infected and healthy uninfected children when adjusting for false discovery rate. To increase sensitivity, we included all genes with unadjusted differential expression $P < 0.05$ (using BloodGen3Module's t-test or limma analyses) and absolute log fold-change greater than 0.5. Even with these liberal thresholds only 3 (limma) or 7 (t-test) out of the 382 modules showed > 15% response, and none had response > 50% (Supplementary Fig. 4). There was no overlap (between the t-test and limma modules) and no clustering at the module aggregate level, indicating that the identified modules are unlikely to be representative of true biological responses to asymptomatic infection. Taken together with the preceding gene-level and modular analyses, these results indicate that asymptomatic *P. falciparum* infection does not elicit a detectable host gene expression response in blood.

Whilst we cannot exclude that much larger studies might detect statistically significant differences in gene expression between

asymptomatically infected and uninfected individuals, our observations have several important implications. First, it appears that asymptomatic parasitaemia occurs when parasite load stays below a threshold necessary to trigger a host response. Second, it suggests that it will be challenging to identify host biomarkers to detect asymptomatic infection, and that sensitive parasite detection methods may be preferable. Third, host blood gene expression may be a promising basis for tests to distinguish whether parasitaemia is causal or an incidental finding in acute illness. Finally, the apparent absence of an active immunoregulatory response during asymptomatic parasitaemia may have implications for the nature and longevity of protection from malaria induced by vaccines versus natural infection.

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Ethical approval and informed consent

Study protocols were approved by the ethics committee of the Ghana Education Service (GES/SHEP/G.23/V.8/18), and the institutional review boards of the Noguchi Memorial Institute for Medical Research (024/14–15) and the London School of Hygiene and Tropical Medicine (14322/15684/17257). Written informed consent was obtained from the parent or guardian of all study participants.

CRediT authorship contribution statement

LEA, GAA, AJC and JCRH conceived the study. DAP collected data and processed samples in the field under the supervision of LEA and GAA. DAP and WJ-W processed samples in the laboratory, under the supervision of AJC and JCRH. DAP, CD, MK, and AN performed the bioinformatic analyses, under the supervision of AJC and JCRH. DAP, CD, AJC and JCRH drafted the manuscript. All authors helped to edit the manuscript. GAA, AJC and JCRH secured funds for the project.

Declaration of Competing Interest

The author declares no competing interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2023.06.013.

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