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Integrative metabolomics and transcriptomics signatures of clinical tolerance to *Plasmodium vivax* reveal activation of innate cell immunity and T cell signaling

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ABSTRACT

Almost invariably, humans become ill during primary infections with malaria parasites which is a pathology associated with oxidative stress and perturbations in metabolism. Importantly, repetitive exposure to *Plasmodium* results in asymptomatic infections, which is a condition defined as clinical tolerance. Integration of transcriptomics and metabolomics data provides a powerful way to investigate complex disease processes involving oxidative stress, energy metabolism and immune cell activation. We used metabolomics and transcriptomics to investigate the different clinical outcomes in a *P. vivax* controlled human malaria infection trial. At baseline, the naïve and semi-immune subjects differed in the expression of interferon related genes, neutrophil and B cell signatures that progressed with distinct kinetics after infection. Metabolomics data indicated differences in amino acid pathways and lipid metabolism between the two groups. Top pathways during the course of infection included methionine and cysteine metabolism, fatty acid metabolism and urea cycle. There is also evidence for the activation of lipoxygenase, cyclooxygenase and non-specific lipid peroxidation products in the semi-immune group. The integration of transcriptomics and metabolomics revealed concerted molecular events triggered by the infection, notably involving platelet activation, innate immunity and T cell signaling. Additional experiment confirmed that the metabolites associated with platelet activation genes were indeed enriched in the platelet metabolome.

1. Introduction

Infections with *Plasmodium vivax* constitute a major public health problem worldwide. *P. vivax* accounted for 41% of estimated malaria cases reported in 2015 outside the African continent [1]. *P. vivax* malaria is characterized by a febrile illness, which may develop into severe symptoms and fatal complications [2]. However, depending on the history of exposure to the parasite, some infections can also remain asymptomatic [3,4]. The molecular mechanisms underlying host responses to *P. vivax* are poorly understood. Controlled human malaria infection (CHMI) trials have become a critically important research tool, and have been used to evaluate the immunization efficacy for *P. vivax* [5–7]. A recent CHMI trial confirmed that previous exposure to *P. vivax* leads to reduced symptoms such as fever and headache [8]. Antibodies from semi-immune individuals reacted to a larger repertoire of *P. vivax* antigens before infection [9], however this was insufficient to control parasite growth after sporozoite challenge [8]. Moreover, symptomatic semi-immune individuals exhibited similar antibody
A. Significant metabolite features across all subjects and time points

B. Heatmap showing metabolite expression levels across baseline, diagnosis, and post-treatment stages.

C. Metabolic pathway diagram including:
- Methionine and cysteine metabolism
- Glyoxylate and dicarboxylate metabolism
- Glycine, serine, alanine and threonine metabolism
- TCA cycle
- Lysine metabolism
- Fatty acid metabolism
- Glycolysis and gluconeogenesis
- Pyrimidine metabolism
- Glycero phospholipid metabolism
- Aminosugar metabolism
- Fatty acid activation
- De novo fatty acid biosynthesis
- Urea cycle/amino group metabolism
- Tyrosine metabolism
- Purine metabolism

D. Graph showing the number of metabolites features (P<0.05) across different stages.

E. Bar graph comparing up-regulated and down-regulated metabolites post-treatment vs baseline.

F. Heatmap depicting metabolite expression levels for the naive group.

G. Heatmap depicting metabolite expression levels for the semi-immune group.

H. Graphs illustrating the changes in metabolite expression levels for tryptophan, kynurenine, glutamate, and N-acetyl-leucine between baseline, diagnosis, and post-treatment stages.
kinetics to that of naïve. Clinical tolerance was associated with antibody reactivity to a smaller subset of antigens [9].

In recent years, important technical developments have emerged allowing the investigation of the human immune responses via high-throughput data [10–16]. These include analyses of human samples using transcriptomics, proteomics, metabolomics, lipidomics and single-cell profiling. Because the human immune system has important differences from animal models, and good animal models are not always available, these studies lead to a detailed understanding of human immunity which is otherwise inaccessible. Metabolomics is global profiling of small molecules in tissues, cells and biological fluids [13]. It captures a “snapshot” of the activity of metabolic processes and molecular phenotypes. Small molecules (metabolites and lipids) not only serve the metabolic need of growth and survival, but also regulate functions of immune cells and systemic signals in infection and inflammation [17–20]. It has been reported that P. falciparum malaria results in altered abundance of plasma metabolites involved in lipid [21], energy [22], and amino acid metabolism [23]. A metabolomics investigation of P. vivax infected patients revealed that their parasite load was associated with heme metabolism and lipid pathways [24].

In this study, we describe the plasma metabolomes from distinct clinical outcomes of a P. vivax CHMI trial, and provide novel insights into associated blood transcriptomes. These are facilitated by our recent development in bioinformatics tools [16,25,26]. Furthermore, the integration of metabolomics and transcriptomics in P. vivax CHMI revealed metabolic processes that were significantly associated with oxidative stress and immunological modalities.

2. Methods

2.1. Ethics statement and clinical trial

The clinical study was conducted at the Malaria Vaccine and Drug Development Center (CIV, Cali) [8]. It was approved by the Institutional Review Boards (IRB) at the CIV and Centro Médico Imbanaco, Cali (Trial Accession #NCT01585077) and the University of Alabama at Birmingham Institutional Review Board (Protocol #X110718014). Written informed consent was obtained at enrollment. Among one-hundred individuals assessed for eligibility at CIV, sixty-nine did not meet inclusion criteria, while fifteen declined to participate. Exclusion criteria included pregnancy; glucose-6-phosphate dehydrogenase (G6PDH) deficiency; positive reactivity for syphilis, HIV, Chagas disease, HTLV 1–2, hepatitis (B – C); or any condition that could increase the risk of adverse outcomes [27,28]. The remaining individuals (n = 16) were allocated into two groups according to the status of previous exposure to P. vivax. Naïve subjects (n = 7) were recruited in Cali (Colombia), a city in which malaria is not endemic. Semi-immune subjects (n = 9) were recruited in Buenaventura, an endemic city for malaria located on the Pacific Coast. The study was exploratory and the sample size was not based on pre-defined effect size but limited by enrollment. Subjects were healthy male and female adults, Duffy-positive (Fy +), 18–45 years of age. The degree of immunity to P. vivax was assessed by clinical history and presence of antibodies against P. vivax blood stages. Anopheles albimanus mosquitoes were reared and infected at the MVDC insectary in Cali, and sporozoite challenge of all subjects was conducted on the same day. Subjects were exposed to bites of 2–4 infected mosquitoes from the same batch. The subjects were monitored daily for clinical manifestations and patent parasitemia. One of the subjects did not develop parasitemia and was excluded from the study. P. vivax infection in challenged subjects was confirmed with thick blood smears (TBS), and retrospectively by RT-qPCR. On the day of parasite detection by TBS, subjects were treated orally with curative doses of chloroquine (1500 mg provided in three doses) and primaquine (30 mg administered once a day for 14 days) [8]. Plasma samples were collected before infection (baseline), on the day of positive blood smear (diagnosis), and three-weeks after treatment.

2.2. Metabolomics analysis and data processing

Liquid chromatography-mass spectrometry (LC-MS) analyses were performed as described [16,29,30]. Briefly, acetonitrile (2:1, v/v) was added to 65 µL of plasma and centrifuged at 14,000 g for 10 min at 4 °C to remove proteins. The supernatant was transferred to an auto sampler vial for LC-MS, using a High Field Q Exact Mass spectrometer (Thermo Fisher), coupled with HILIC liquid chromatography. Mass spectral data was acquired with positive electrospray ionization and the full scan of mass-to-charge ratio (m/z) ranged from 85 to 1275 at a resolution of 120,000. Each sample was run in triplicate in batches of 20 samples. Peak detection, noise filtering, m/z, and retention time alignment, and feature quantification were performed with aplCMS [31] and xMSanalyzer [32]. Each metabolite feature is defined by m/z and retention time, with intensity values associated with each replicate. Data were averaged among replicates, log2 transformed and normalized by the mean. Only features detected in more than 75% of all samples (4236) were used in further analysis. The reporting of metabolite annotation adheres to the five confirmation levels in metabolomics literature [33,34]. Level 1 annotation applies to the metabolites confirmed by matching both m/z (mass accuracy under 10 ppm) and retention time to that of authenticated chemical standards, previously characterized in our laboratory (Supplemental Table 1). Additional putative annotation was performed by m/z matching to KEGG database (mass accuracy under 10 ppm – annotation level 3) [35]. The mumichog software (version 1.0.7) was used for metabolic pathway analysis (mass accuracy under 10 ppm) [25]. The raw metabolomics data have been made publicly available in the MetaboLights repository (https://www.ebi.ac.uk/metabolights/, study ID MTBLS665). A list of all publicly available MaHPIC datasets is at http://plasmodb.org/plasmo/mahpic.jsp.

2.3. Platelet isolation

Collection of human platelets was approved by the University of Alabama at Birmingham Institutional Review Board, and performed as described [36]. Briefly, platelet-rich plasma, obtained from individual donors from the blood bank at the University of Alabama at Birmingham, was centrifuged at 1500 g for 10 min, and washed with PBS containing prostaglandin I2 (1 µg/ml). Platelets were diluted in DMEM assay buffer (DMEM with 1 mM pyruvate, 5.5 mM glucose, 4 mM L-glutamine, pH 7.4), and incubated for 3 h at 37 °C. After washing with cold PBS, platelets were spiked with internal isotope standards, and distributed to a final concentration of 300 × 10^6/150 µL of acetonitrile. After incubation on ice for 15 min, proteins were removed by
Fig. 2. Differential metabolite abundance between naïve and semi-immune subjects. A, Significant metabolite features differing between naïve and semi-immune subjects. Normalized intensity values were used to identify significant features at baseline. Baseline normalized intensity values were used to identify significant features at diagnosis and post-treatment time points. Limma moderated t-statistic was used. B, Two-way hierarchical clustering based on differentially abundant metabolite features between naïve and semi-immune subjects at baseline, diagnosis/baseline and post-treatment/baseline. C, Boxplots for uric acid (baseline) or indole-3-acetaldehyde (post-treatment/baseline). Significance level is shown as *, p < 0.05. D, Summary of metabolic pathways enriched by significant metabolite features. Mumichog software was used for pathway enrichment using the top 200 most significant metabolite features for each statistical comparison between time points or immune status. Only pathways represented by at least four metabolite features and enriched at p < 0.05 are shown. E, Abundance kinetics for plasma glutamine. Significant metabolite features were identified by ANOVA with repeated measures. Tukey’s multiple comparisons test was used in additional statistics. Significance levels are shown as *, p < 0.05. F, Correlation between plasma glutamine and blood cell glutaminase (GLS) expression assayed by RNA-seq.

3. Results

This CHMI study included two groups of participants: one group naïve and the other semi-immune to P. vivax infection and which was shown to be more tolerant to symptoms of P. vivax infection [8]. The goal of this study is to delineate the molecular differences between the naïve group and the semi-immune group. To this end, we first report the metabolic changes during the time course of infection (Fig. 1), then compare the two groups by their plasma metabolomics (Fig. 2) and blood transcriptomics (Fig. 3). The integration of metabolomics and transcriptomics revealed significant associations induced by the infection (Fig. 4), and we further validate the platelet activation pathway suggested by the integrative analysis (Fig. 5).

3.1. Plasma metabolomic signatures along the course of Plasmodium vivax CHMI

This CHMI study using P. vivax was monitored as a time course: baseline was taken as 2 days prior to infection; parasites were detected in the subjects’ blood around 10 days after infection, and treatment was started on this same day of diagnosis. The treatment included three doses of chloroquine and 14 days of primaquine. Plasma samples from three time-points, baseline, diagnosis and post-treatment, were analyzed by untargeted high-resolution metabolomics. This resulted in detection of over 8000 metabolite features. After filtering for missing values, 4236 were retained for statistical analysis. The abundance of 964 metabolite features was significantly altered across the three time-points (p < 0.05; 0 at FDR < 0.1; Fig. 1A-B), irrespective of the immune status of subjects, while one-way hierarchical clustering revealed high variability among the subjects (Fig. 1B). To evaluate the metabolic pathways underlying these significant metabolite features, we used mumichog, a software specifically designed for untargeted metabolomics [25], which has gained increasing popularity [34,39,40]. The results indicate that significant features are enriched for pathways involved in amino acid metabolism, carbohydrate metabolism, energy metabolism, lipid metabolism and nucleotide metabolism (Fig. 1C). Examples of these significant metabolites include carnitine (Fig. 1D), methionine and oxoproline (Supplementary Figure 1A).

The metabolites associated to time-points suggest that a greater metabolic shift was seen post-treatment than at the time of diagnosis (Fig. 1B). Indeed, when each time-point of diagnosis or post-treatment was compared to baseline, the greatest effects in the plasma metabolome of all subjects occurred post-treatment (Fig. 1E). This observation remained true when the naïve group and semi-immune group were analyzed separately (Fig. 1E, F-G). Specific metabolites, however, appeared to be significant in each of the groups. For the naïve subjects, these include tryptophan, kynurenine and glutamine (Fig. 1H, Supplementary Table 1). For the semi-immune subjects, these include glutamate, N-acetyl-leucine and pantothenate (Fig. 1H, Supplementary Table 1). Overall, these data suggest that the treatment drugs had a major effect on the host metabolome. In addition, the infection also induced metabolic changes in peripheral blood by the time of diagnosis, which was more pronounced in the semi-immune group (Fig. 1E, G).
A

Diagnosis vs Baseline

(Baseline)

(Diagnosis/Baseline)

B

Enrichment plot: ENRICHED IN NEUTROPHILS (M37: 53)

C

Type I interferon response

Inflammatory response

Myeloid cells

B lymphocytes

T lymphocytes

Other functional processes

D

Diag./Basel. M127 (interferon genes)

Diag./Basel. M37.1 (neutrophil genes)

Diag./Basel. M11.1 (coagulation genes)

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3.2. Naïve and semi-immune subjects exhibit distinct metabolic profiles in plasma

To investigate the metabolic difference between naïve and semi-immune subjects, we compared the two groups at baseline, diagnosis normalized to baseline and post-treatment normalized to baseline. At diagnosis/baseline, 126 metabolite features differed significantly between the two groups (p < 0.05), while greater numbers were seen at baseline and post-treatment (Fig. 2A, B). This does not necessarily indicate that the baseline difference was greater, because it was removed in the comparisons after infection by subtracting the baseline values. The differential metabolites at three time-points are given in Supplementary Table 1. A few examples include uric acid and indole 3-acetaldehyde (Fig. 2C).

We compared the significant pathways over the time course in each group (Fig. 2D, left), and between the two groups at each time point (Fig. 2D, right). To be consistent in all six analyses, the 200 most significant metabolite features were used for pathway test in each case. In the time course analysis, it appears that most pathways were driven by changes relative to the naïve group, most notably methionine and cysteine metabolism and glycine, serine, alanine and threonine metabolism. The semi-immune group exhibited a significant change in urea cycle/amine group metabolism, linoleate metabolism and arginine and proline metabolism. When the two groups were compared to each other, few pathways showed significant enrichment, mostly at post-treatment. Linoleate metabolism, however, reached significance again at the time of diagnosis (Fig. 2D, Supplementary Fig. S1B). Interestingly, the 3 major metabolite features related to linoleate are derived from lipid peroxidation reactions. Epoxynonanal is an oxidation product that is likely to be derived from the decomposition of the lipid hydroperoxide 9(s)-HPDPE. The lipid peroxidation can be formed either enzymatically through the action of lipoxygenase or through non-specific lipid peroxidation and the 13-OxoODE is known to also be derived from the enzymatic decomposition of lipid peroxides [41]. This together with significant changes in the lipid metabolites prostaglandin and eicosapentaenoic acid (Supplementary Table 1) indicates a difference in inflammatory response at the time of diagnosis between two groups. Interestingly, although many metabolite features differed at baseline, they did not significantly match known metabolic pathways (Fig. 2D).

The difference in urea cycle/amine group metabolism and arginine and proline metabolism is exemplified by glutamine (Fig. 2E). Glutamine is converted to glutamate by glutaminase (GLS), and is a major precursor for the de novo synthesis of arginine in humans [42]. Both arginine and glutamate are involved in urea cycle. Arginine is depleted during P. vivax malaria, and this reduction is associated with endothelial dysfunction [43]. The level of glutamine was examined in P. falciparum malaria before with varying conclusions [44,45]. Our data indicate that glutamine levels remained relatively stable in semi-immune subjects, but decreased after infection in the naïve subjects (Fig. 2E). Glutamine levels exhibited a clear and inverse correlation with GLS expression level assayed by RNAseq (Fig. 2F).

3.3. Transcriptomic programs differentiate naïve and semi-immune subjects

The blood transcriptomics of this P. vivax CHMI study was obtained by RNAseq using mRNA from whole blood at baseline and at diagnosis. The initial analysis was described previously [37]. Consistent with the earlier publication, significant changes in gene expression occurred after infection, but no apparent difference was seen between naïve and semi-immune subjects at gene-level analysis (Fig. 3A). The recently developed new tool, Blood Transcription Modules (BTM), demonstrated higher sensitivity of capturing immunological events from blood transcriptomics [26]. Using BTM with the well-established GSEA software indeed shows significantly differential gene modules between the two groups. For example, a neutrophil gene module was significantly higher in semi-immune compared to naïve subjects (Fig. 3B).

A summary of significantly enriched BTMs is shown in Fig. 3C. Both naïve and semi-immune groups exhibited up-regulation of type I interferon response and dendritic cell activation, and decrease of neutrophil signals after infection (Fig. 3C, left three columns, Fig. 3D). Direct comparison of naïve and semi-immune subjects revealed detailed difference in their immune responses (Fig. 3C, right two columns). While the semi-immune subjects showed a higher level of B lymphocytes at baseline, at the time of diagnosis, they were highly enriched for modules corresponding to an inflammatory response mediated by myeloid cells. This is consistent with the inflammatory lipids on linoleate pathway at diagnosis from plasma metabolomics data (Fig. 2D, Supplementary Figure 1B). The T cell modules showed a lower level of signals in these subjects, but this can be attributed to homeostasis that offsets the higher level of myeloid cells. Both volunteer groups had decreased number of neutrophils after infection, but the neutrophils in the semi-immune group still significantly outnumbered those in the naïve group (Fig. 3C, D). The semi-immune group also displayed higher activity in other processes, such as cell cycle and blood coagulation (Fig. 3C, D). Taken together, these data indicate that the semi-immune group launched a stronger innate activation at the time of diagnosis, which then contributed to their improved control of symptoms after P. vivax infection.

3.4. Infection with Plasmodium vivax induces concerted metabolic and transcriptional responses

Although the transcriptomics was measured from circulating immune cells and metabolites from plasma, the above data suggest that they are not isolated from each other. Recent literature also indicates that metabolites are integral signals in immune responses [16,17,46]. We therefore set out to investigate the associations between transcriptomics and metabolomics data. We adopt here a method recently published by our group [16]. This method combines meaningful dimension reduction with PLS regression. The transcriptomics data were collapsed into BTM clusters, and metabolomics data were collapsed into metabolite clusters. The PLS regression accounts for different variance structures of different technologies. The statistical significance of those associations was evaluated by permutation [16]. This has the added benefit on statistical power because only consistent signals in both data types will reach significance.

At baseline, only one significant association between
A) **Baseline**

B) **Diagnosis/Baseline**

- Chemokines and T cell signaling
- IFN signature and innate cell activation
- Platelet complement activation

**Metabolites**

**Genes**

- BTM cluster
- Metabolite cluster

C) **BTM cluster 23**

- Transcription elongation, RNA polymerase II (M234)
- Immune regulation – monocytes, T and B cells (M57)
- RA, WNT, CSF receptors network (monocyte) (M23)
- Chemokine cluster (I) (M27.0)
- Signaling in T cells (I) (M35.0)
- AP-1 transcription factor network (M20)
- Targets of FOSL1/2 (M0)
- Putative targets of PAX3 (M89.1)
- Putative targets of PAX3 (M89.0)

D) **M38, chemokines and receptors**

E) **Pathways enriched in Metabolite cluster 3**

- Aspartate and asparagine metabolism
- Urea cycle/amino group metabolism
- Carnitine shuttle
- Biotin metabolism
- Glycine, serine, alanine and threonine metabolism
- Arginine and Proline metabolism
- Lysine metabolism
- Tyrosine metabolism
- Butanolate metabolism
- Pyrimidine metabolism
- Tryptophan metabolism
- Glycerocephospholipid metabolism
- Drug metabolism - cytochrome P450

F) **Metabolite cluster 3**

- Infected volunteers

G) **Tyrosine**

- m/z - 182.0818
- RT - 101
- M+H [1+]

- Log Fold Change (Diag./Baseline)

- Naive
- Semi-immune

H) **Correlation between M38 genes and metabolites**

- Naive
- Semi-immune

- CCR5
- CXCR4
- CXCR4 (Diag./Baseline)
- Tyrosine (Diag./Baseline)
- Serine (Diag./Baseline)
- Glutamate (Diag./Baseline)
- IL-8 (Diag./Baseline)
- IL-8 (Diag./Baseline)
- IL-8 (Diag./Baseline)

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transcriptomics and metabolomics was detected (Fig. 4A), not surprisingly, because of the small cohort and the contribution of transcriptomics and metabolomics coming from different compartments (cells and plasma, respectively). However, in stark contrast, infection with *P. vivax* induced many significant associations between the two data types (Fig. 4B, 26 edges with *p* < 0.001, 88 edges with *p* < 0.05). These associations center on three transcriptomic hubs (BTM clusters): IFN signature and innate cell activation gene modules, chemokines and T cell signaling, and platelet and complement activation (Fig. 4B). As type I IFN response is important in the transcriptomic signature (Fig. 3C, D), it's expected to be concordant with several other innate pathways (Supplementary Figure 2A). The hub on Chemokines and T cell signaling (BTM cluster 23) is further listed in Fig. 4C, and member genes in Fig. 4D. This overlaps with the inflammatory response and myeloid cell signature in Fig. 3C (RA, WNT, CSF receptors and network), which differed between the naïve and semi-immune groups. Although other gene modules share a similar profile, not all modules are significant in Fig. 3C, as different statistics is applied here to identify the most association with metabolites. One of the metabolite clusters associated with this hub is exemplified in Fig. 4E, and consisted of 205 metabolite features of similar profiles. The members in this cluster are related to amino acid metabolism (Fig. 4F), and the examples of tyrosine and serine are shown in Fig. 4G. The associations between amino acids (tyrosine, serine and glutamate) and M38 members CXCR4 and IL8 are further illustrated in Fig. 4H.

### 3.5. Metabolomic confirmation of platelet involvement in response to *P. vivax* infection

The other significant transcriptomic hub relates to platelet and complement activation (Fig. 4B). This cluster consists of genes mostly related to platelet activation, blood coagulation and SRF (serum response factor) signaling (Fig. 5A). Their expression level is higher in the semi-immune group at the time of diagnosis (Fig. 5B), though both naïve and semi-immune groups exhibited platelet depletion after *P. vivax* infection (Fig. 5C), consistent with previous literature [47]. Associated with this hub are many metabolite clusters encompassing 881 features (*p* < 0.05 Figs. 4B, 5D). Platelets are now increasingly recognized as immune cells [48,49]. Platelets mediate the agglutination of *P. falciparum*-infected erythrocytes [50], and play a protective role by killing intraerythrocytic *P. falciparum* parasites [51]. However, *P. vivax* infected cells do not seem to adhere to platelets [52], suggesting distinct platelet-mediated effector mechanisms [53]. The quantitative difference in platelet signals appears to be a key part in the different responses to *P. vivax* infection between the naïve and semi-immune groups.

The significant association between platelet activation modules and metabolomics data (Fig. 5D) raised the possibility that the plasma metabolomics was strongly influenced by platelets. This is possible because platelets remain in the plasma layer after whole blood is centrifuged and processed. To test this hypothesis, we assessed platelets from healthy donors and performed metabolomics analysis on the same LC-MS platform. Among the 881 metabolite features associated to BTM cluster 8 (*p* < 0.05; Fig. 5E), 309 were found in the platelet metabolome (Fig. 5F). We then compared each of these metabolite clusters against the 200 most abundant m/z peaks in platelets, and they were clearly enriched in platelet metabolites (Fig. 5G). The enrichment p-value for metabolites in cluster 8 was under 10<sup>−23</sup> (hypergeometric test). These data confirm that the plasma metabolomics captured a strong signal from platelets. Furthermore, our integration method was successful in identifying this concerted event between blood transcriptomics and plasma metabolomics. Taken together, these data indicate that the *P. vivax* infection triggered a broad array of concerted molecular programs in systemic metabolites and blood cells.

### 4. Discussion

Clinical tolerance to re-infections with *Plasmodium* has been well documented in endemic regions for malaria [54]. This phenomenon is likely driven by the coordinated activity of multiple biological processes, which are not completely understood. Oxidative stress is reported part of the infection and pathogenesis of malaria [55]. This is supported by the many amino acid and lipid pathways identified in this study. Moreover, redox is a key part of immune response in both pathogen suppression and host signaling [56,57]. Recent studies show that combined metabolomics and transcriptomics analyses provide sensitive approaches to link oxidative stress to biologic responses [58]. Here we demonstrate that, compared to naïve subjects, those with previous history of *P. vivax* malaria exhibited distinct plasma metabolic signatures during a CHMI trial. Notably, naïve subjects exhibited greater perturbations on the plasma metabolome in a time course analyses. However, metabolic signatures from semi-immune subjects showed a more significant response after repeated exposure to *P. vivax*. The semi-immune subjects in this study developed minor to no symptoms [8], and complete tolerance often requires several episodes of malaria [54]. Thus, a short-lived immunological memory might account for differences between clinical outcomes [54]. These subjects were recruited from different regions in Colombia and may exhibit slightly differing life-styles and while these could influence the metabolic phenotypes it is unlikely they make a major contribution to the differences in the patient cohorts reported here. Nevertheless, our data supports the hypothesis that clinical tolerance to *P. vivax* results in a distinct metabolic profile, especially linoleate metabolism.

During the CHMI trial, several amino acids and related metabolites displayed differential kinetics, including glutamine, kynurenine and tryptophan. Kynurenine’s abundance increased with concomitant depletion of tryptophan in plasma of naïve subjects after *P. vivax* infection but returned to baseline levels after treatment. Both tryptophan and kynurenine’s abundance remained unaltered in semi-immune subjects (Fig. 1H). The conversion of tryptophan to kynurenine is catalyzed by indoleamine 2,3 dioxygenase (IDO), and the inhibition of IDO was shown to protect mice from lethal malaria parasite infection [59]. Therefore, tryptophan metabolism may play a role in the pathogenesis of *P. vivax* malaria. Kynurenine contributes to arterial-vessel relaxation during malaria inflammation in mice [60], and could also be involved with endothelial dysfunction and hypotension in severely ill *P. vivax* patients. Of note, a higher proportion of naïve subjects had severe headaches [8]. While caused by *P. falciparum*, children with cerebral malaria were shown to possess elevated levels of kynurenine-derived metabolites in their cerebrospinal fluid [61]. Moreover, inhibition of this pathway prolongs survival in a mouse model of cerebral malaria [62]. We hypothesize that this pathway may be a target to reduce symptoms of *P. vivax* malaria.
E

Associated metabolites

BTM cluster 8

A

- platelet activation & blood coagulation (M199)
- complement activation (I) (M112.1)
- cytoskeleton/actin (SRF transcription targets) (M145.1)
- integrin cell surface interactions (I) (M1.0)
- platelet activation – actin binding (M196)
- cell movement, Adhesion & Platelet activation (M30)
- platelet activation and degranulation (M85)
- cytoskeletal remodeling (enriched for SRF targets) (M34)
- G protein coupled receptors cluster (M155)
- enriched in myeloid cells and monocytes (M61)
- G protein mediated calcium signaling (M159)
- TBA (M104)
- enriched in membrane proteins (M124)
- cytoskeleton/actin (SRF transcription targets) (M145.0)

B

- PECAM1 (Diag./Basel.)
- SIRPA (Diag./Basel.)
- SERPINA1 (Diag./Basel.)

C

- Platelet count (x10^11/uL)

(caption on next page)
The semi-immune group also differed from the naïve group in inflammatory lipids related to the linoleate pathway. These lipid mediators constitute a complex bioactive network with potent inflammatory and regulatory properties and interestingly decomposition products from lipoygenase-derived lipid peroxides [63]. In addition to the lipid peroxidation products produced by the enzymatic action of lipoygenases, cyclooxygenase products are also produced. For example, prostaglandin E2 (PGE2) inhibits type I interferon response to Mycobacterium tuberculosis, which is crucial for protection [64]. P. vivax infection induces the transcriptional activity of type I interferon pathway [37], while PGE2 levels are reduced during severe P. vivax malaria [65]. Our metabolite data corroborate the transcriptional response: at the time of malaria diagnosis, the transcriptional profiles of semi-immune subjects mainly reflect a response mediated by myeloid cells when compared to naïve subjects. Thus, the tolerance to clinical symptoms might involve more than the memory developed by lymphocytes, and may also rely on trained innate immune cells. This concept emerged from observations that innate immunity can be crucial for re-infections [66]. Indeed, trained immunity involves epigenetic and metabolic reprogramming of innate immune cells [66] and is induced by P. falciparum infection [67]. Our data suggest that metabolic reprogramming of myeloid cells could have a substantial effect on the development of clinical tolerance to re-infection with P. vivax, in which platelets play a significant role. Recent work demonstrated that repeated exposures to P. vivax results in higher frequencies of classical memory B cells, reduction of atypical memory B cells, and increased levels of P. vivax-specific IgG [68]. Moreover, women exposed to P. vivax exhibited a higher proportion of atypical memory B cells [69]. Thus, the extensive associations with BTMs related to B cells at baseline for semi-immune, but not naïve subjects, indicate that clinical tolerance to P. vivax malaria is accompanied by increased circulation of memory B cells.

To our knowledge, this is the first study to integrate host metabolomics and transcriptomics in the context of human malaria research. The major limitation of this study is the small cohort size. However, this will be a common challenge as we move towards precision medicine approaches to big data. It would be useful to test the hypotheses in a separate cohort, a larger population or appropriate animal models. Without any of those, one way to address the challenge is the integration of orthogonal and intensive data points – the integration of metabolomics and transcriptomics here enhanced the statistical power. Because of the limitation of sample size, little significant association was found between the orthogonal datasets at baseline. However, stronger signals elicited by the infection pinpointed to a concerted network, hinging on gene modules related to platelet activation, type I interferon and innate immunity, and for chemokines and T cell signaling. Among the associated metabolic pathways, linoleate and glycerophospholipid metabolism were also associated with the transcriptional activity of innate immunity, T and B cells in response to a live attenuated viral vaccine in humans [16]. By measuring the platelet metabolome separately, we were able to confirm that the plasma metabolites associated with platelet activation genes in blood cells were indeed highly enriched in platelets. Overall, these results indicate that the immune response to P. vivax infection is tightly associated with host metabolic responses. A better understanding of their interplay will be useful for designing novel therapeutic interventions and vaccines.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at 10.1016/j.redox.2018.04.011.

References


Fig. 5. Molecular signatures of platelet activation in P. vivax infection. A, Heat map of BTMs’ activity for each P. vivax infected subject, included in BTM cluster 8 (Platelet, complement activation) identified by integrative metabolomics and transcriptomics as described in Fig. 4B. B, Boxplots of platelet activation genes PECAM1, SIRPA, SERPINA1 expression assayed by RNA-seq. C, Platelet counts from whole blood at baseline and diagnosis time points. Significance levels are shown as * , p < 0.05; **, p < 0.01. D, Highlighted associations between metabolites and gene cluster 8, as described in Fig. 4B. E, Metabolite clusters associated with gene cluster 8 exemplified as heatmaps. F, Venn diagram showing common m/z peaks detected in platelets derived from healthy donors and plasma metabolite clusters associated with gene cluster 8. G, Metabolite cluster m/z peak over-representation in platelet-enriched metabolites.


