Integrated pathogen load and dual transcriptome analysis of systemic host-pathogen interactions in severe malaria

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Overline: Malaria
One sentence summary: Host and parasite RNA-sequencing is combined with parasite load estimates to reveal mechanisms associated with human severe malaria.

Abstract

The pathogenesis of infectious diseases depends on the interaction of host and pathogen. In Plasmodium falciparum malaria, host and parasite processes can be assessed by dual RNA-sequencing of blood from infected patients. Here we performed dual transcriptome analyses on samples from 46 malaria-infected Gambian children to reveal mechanisms driving the systemic pathophysiology of severe malaria. Integrating these transcriptomic data with estimates of parasite load and detailed clinical information allowed consideration of potentially confounding effects due to differing leukocyte proportions in blood, parasite developmental stage, and whole-body pathogen load. We report hundreds of human and parasite genes differentially expressed between severe and uncomplicated malaria, with distinct profiles associated with coma, hyperlactatemia, and thrombocytopenia. High expression of neutrophil granule-related genes was consistently associated with all severe malaria phenotypes. We observed severity-associated variation in the expression of parasite genes which determine cytoadhesion to vascular endothelium, rigidity of infected erythrocytes, and parasite growth rate. Up to 99% of human differential gene expression in severe malaria was driven by differences in parasite load, whereas parasite gene expression showed little association with parasite load. Co-expression analyses revealed interactions between human and P. falciparum, with prominent co-regulation of translation genes in severe malaria between host and parasite. Multivariate analyses suggested that increased expression of granulopoiesis and interferon-γ related genes, together with inadequate suppression of type-1 interferon signalling, best explained severity of infection. These findings provide a framework for understanding the contributions of host and parasite to the pathogenesis of severe malaria and identifying targets for adjunctive therapy.

Introduction
Most studies of infectious disease pathogenesis focus on either host or pathogen, despite the fact that outcome is determined by their interaction. Dual RNA-sequencing has been developed as a method for transcriptomic assessment of such interactions (1, 2), although it has not been widely applied to study systemic infection in humans. In malaria, the pathogenic stage of the parasite is restricted to blood where important interactions between parasites and host leukocytes can be assessed by dual RNA-sequencing (3). The blood is also the conduit for systemic responses to infection, and gene expression in blood will reflect the inflammatory and metabolic milieu to which leukocytes and parasites are exposed.

*Plasmodium falciparum* malaria is one of the most important infectious diseases affecting humans (3). Most malaria deaths occur in children, in whom three major syndromes are associated with increased risk of death and distinguish severe malaria from uncomplicated malaria: 1. cerebral malaria (manifesting as coma), 2. hyperlactatemia / acidosis (often manifesting as deep breathing), and 3. severe anemia (3-6). These are usually accompanied by the laboratory finding of low platelet count (thrombocytopenia) (7). Severe malaria syndromes can occur in isolation or in overlapping combinations (4), and mortality is highest when cerebral malaria and hyperlactatemia / acidosis coexist (4). Severe malaria is most likely when there is a high parasite load (8-11) and numerous accompanying pathophysiological derangements have been described (4, 5, 12), broadly arising from inflammation, vascular endothelial dysfunction and parasite sequestration (accumulation of parasitized erythrocytes in small blood vessels which obstruct blood flow) (4, 13). These mechanisms interact with one another, so defining their individual contributions to specific features of severe malaria is challenging (13, 14).

The host immune response is a major determinant of outcome in rodent models of severe malaria (15, 16) and it has long been supposed that an excessive host response may also contribute to some forms of human severe malaria (17, 18). A similar concept exists to explain severity in other infections such as bacterial sepsis, Ebola and Respiratory Syncytial Virus (19-21), however direct evidence is often lacking and the confounding effect of pathogen load on the magnitude of the host
response is rarely quantified. Controlled human infection models have provided insights into the
relationship between pathogen load and early immune responses to infection (22) but cannot be
extended to investigate severe disease. To better understand the pathogenesis of severe infection, a
systemic, integrated view of host-pathogen interaction accounting for variation in pathogen load is
required.

The feasibility of host and parasite dual RNA-sequencing in malaria has been demonstrated in
individuals with uncomplicated malaria (23). Here, we extend the application of dual RNA-
sequencing to infectious disease pathogenesis by integrating gene expression analysis with detailed
clinical and laboratory data that characterise the systemic pathophysiology of severe malaria. We
further refine the analysis by accounting for three major confounders which may vary within and
between severity groups. This allows us to characterise human and parasite differential gene
expression between severe and uncomplicated malaria and the role of parasite load in determining
the host response. These data provide a unique insight into host-pathogen interactions associated
with severity of infection in humans and reveal new perspectives on the likely pathogenic
mechanisms of human severe malaria.

Results

Dual RNA-sequencing and adjustment for cellular heterogeneity

We performed dual RNA-sequencing on whole blood, collected prior to treatment, from 46 Gambian
children with *P. falciparum* uncomplicated malaria (*n* = 21) and severe malaria (*n* = 25) (Table 1).
These children were recruited from a region with relatively low malaria transmission and consistent
with this epidemiology, (4) the severe malaria group contained children with cerebral malaria,
hyperlactatemia, or a combination thereof, but no cases of severe anemia. After exclusion of
parasite var, rifin, stever (14) and other highly polymorphic regions for which reference genome-
based mapping is not possible, we obtained medians of 26.6 million human uniquely mapped reads
from each subject (26.6 million severe malaria, 26.7 million uncomplicated malaria, Mann-Whitney *P*
0.913) and 9.61 million parasite uniquely mapped reads (10.3 million severe malaria, 5.03 million
uncomplicated malaria, Mann-Whitney \( P = 0.346 \) (Fig 1A). We detected expression of 12253 human
and 3880 parasite genes. Commensurate with the high parasitemias seen in these children (Table 1),
parasite read depth was considerably greater than in a previous study of Indonesians with
uncomplicated malaria (23).

Systemic infection provokes changes in blood leukocyte subpopulations which can dominate
changes in gene expression and confound their interpretation (24). Amongst our study subjects
there were significant differences between clinical groups in the proportions of neutrophils (Kruskal-
Wallis \( P = 0.01 \)) and neutrophil counts (Kruskal-Wallis \( P = 0.05 \)) in blood (Table 1). We performed
gene signature-based deconvolution (25) to estimate heterogeneity in the contribution of the major
leukocyte subpopulations to the RNA in our samples (Fig 1B, fig S1). Parasite gene expression in vivo
is also dominated by the mixture of parasite developmental stages at the time of sampling because
there is phasic variation in gene expression (26) and total RNA content increases during the
intraerythrocytic developmental cycle (27). Therefore we also applied the deconvolution approach
with reference gene signatures derived from highly synchronous parasite cultures (26, 28) to identify
the contribution of parasites at different developmental stages (Fig 1C). As the method was
developed and validated for distinct human cell types, we confirmed the effectiveness of estimation
of parasite developmental stage mixture by comparison with previously proposed stage-specific
marker genes (29), and by assessment of performance in synthetic datasets of known composition
(fig S2). We compared relative contributions of parasite developmental stages between severe and
uncomplicated malaria samples and observed a trend towards greater contributions from late stage
asexual parasites and gametocytes in children with severe malaria (Fig 1D), consistent with previous
reports (30).

In order to remove the confounding effect of heterogeneity in leukocyte and parasite mixtures we
adjusted gene expression values for the proportions of detected cell types, essentially allowing us to
compare gene expression as if all subjects had the same leukocyte and parasite population compositions. Adjustment for heterogeneity in the mixture of leukocytes and parasite developmental stages improved segregation of severe and uncomplicated malaria cases (Fig 1E, F; multivariate ANOVA for human gene expression $P = 0.0013$ and $P = 0.00012$, and for parasite gene expression $P = 0.0049$ and $P = 0.00019$, before and after adjustment, respectively). Therefore we used adjusted gene expression values for all subsequent analyses.

**Gene expression associated with severe malaria and parasite load**

To identify differentially expressed genes between clinical groups, and to identify gene expression associated with continuous variable severity features, we used a generalised linear model approach incorporating leukocyte populations or parasite stage as covariates, and we considered a false discovery rate (FDR)-adjusted $P < 0.05$ as significant. Considering all subjects, there were 770 human significantly differentially expressed genes between severe and uncomplicated malaria (Fig 2A, table S1). Genes more highly expressed in severe malaria versus uncomplicated malaria most notably included *MMP8* (matrix metallopeptidase 8), *OLFM4* (olfactomedin 4), *DEFA3* (defensin A3), and *ELANE* (neutrophil elastase), all encoding neutrophil granule proteins (31). Given our adjustment for cellular heterogeneity, these results likely reflect a true increase in transcription of these genes rather than a greater proportion of neutrophils in the blood, and differences remained when absolute neutrophil counts were multiplied by expression (Fig 2B). We performed Gene Ontology (GO) analyses to better understand the biological functions of the differentially expressed genes (Fig 2C) and identified enrichment of co-translational protein targeting, cell motility and immune response functions (table S2). We used Ingenuity Pathway Analysis (Qiagen Bioinformatics) to predict upstream regulators of the differentially expressed genes, and colony stimulating factor 3 (CSF3, also known as granulocyte colony stimulating factor, GCSF), Fas cell surface death receptor, and Prostaglandin E receptor 2 signalling were amongst the most over-represented (table S3). We repeated similar analyses to identify and interpret genes differentially expressed between
uncomplicated malaria and different clinical phenotypes of severe malaria (fig S3, tables S1&S2), which revealed many consistent associations but also some notable differences. For example, the number of differentially expressed genes substantially increased when comparing the subgroup of subjects with cerebral malaria plus hyperlactatemia (the most severe phenotype, n = 12) vs uncomplicated malaria (n=21), possibly reflecting their greater severity and homogeneity of disease (table S1, figure S3). The most highly expressed genes in these patients relative to uncomplicated malaria included genes for neutrophil granules and heat shock proteins.

In order to gain a greater insight into specific pathophysiological processes, we examined the quantitative association of gene expression with clinical and laboratory parameters (table 1) which characterise specific aspects of severe malaria pathophysiology: consciousness level (using the Blantyre Coma Scale, BCS), blood lactate concentration, platelet count and hemoglobin concentration (table S4). 738 genes were significantly associated with BCS level (using ordinal regression with FDR $P < 0.05$, table S4) and decreasing consciousness level (lower BCS) was associated with both higher expression of genes involved in the cell cycle and lower expression of genes involved in MHC class I antigen presentation and interferon-γ (IFN-γ) signalling (Fig 2C, table S2). Predicted upstream regulators included estrogen receptor 1 and transglutaminase 2 (table S3).

1012 human genes were associated with lactate concentration (table S4), amongst which immune response pathways were again prominent, but a negative association between lactate and type 1 IFN signalling was particularly notable (Fig 2C, table S2), and the most strongly predicted upstream regulators were IFN-γ, IFN-α, and TNF (table S3). 178 genes were associated with platelet count (table S4) and the most enriched pathways differed considerably from those in the preceding analyses (Fig 2C, table S2), with nucleosome assembly (predominantly histone genes), coagulation, and response to wounding genes all negatively correlated, and the most strongly predicted upstream regulators being IL13, RB transcriptional corepressor 1, and IL1RN (table S3). No human genes correlated with hemoglobin concentration. Taken together, these results identify common
transcriptional features of severe malaria but also implicate distinct mechanisms underlying the
different pathophysiological processes which can occur in severe malaria.

There were 236 parasite genes differentially expressed between severe and uncomplicated malaria
(Fig 2D, table S5). The parasite gene with highest expression in severe relative to uncomplicated
malaria was PF3D7_1016300, a gene which encodes a glycophorin binding protein (GBP) expressed
in the cytoplasm of infected erythrocytes and which influences adhesion and rigidity of the red cell
(32, 33). The most down-regulated parasite gene (with known function) in severe relative to
uncomplicated malaria was PF3D7_1222600 which encodes the AP2 domain transcription factor
AP2-G. This protein controls the balance between gametocytogenesis and asexual replication, and
knockout of the orthologue in P. berghei ANKA enhances the in vivo asexual parasite growth rate
(34, 35). As with human gene expression, there were more differentially expressed genes in the
comparison of uncomplicated malaria vs. the cerebral malaria plus hyperlactatemia group (fig S3,
table S5). Here the most differentially expressed parasite genes included PF3D7_0202000 (knob-
associated histidine rich protein), PF3D7_1016300 (GBP), PF3D7_0201900 (erythrocyte membrane
protein 3), and PF3D7_0424600 (PHIST-b protein), all of which encode proteins that interact with the
erthrocyte cytoskeleton to influence cytoadhesion and deformability of the infected erythrocyte,
making them plausible determinants of severity (33). Parasite genes differentially expressed in
severe compared to uncomplicated malaria were enriched in specific biological functions including
RNA processing, protein transport, and hemoglobin catabolism (Fig 2E, table S6).

445 parasite genes were significantly associated with BCS level (using ordinal regression with FDR \( P < 0.05 \)), and those most significantly associated with lower BCS were PF3D7_0919800 (TLD domain-
containing protein), PF3D7_1133700 (FHA domain-containing protein), and PF3D7_1408200 (AP2
domain transcription factor AP2-G2), the latter two being important determinants of asexual
parasite growth rate (35, 36) (table S7). The most enriched functions associated with BCS included
transport, hemoglobin catabolism and prenylation (Fig 2D). 100 parasite genes associated with
lactate (table S7). The most significant (FDR \( P = 2.4 \times 10^{-6} \)) was PF3D7_0201900 (encoding erythrocyte
membrane protein 3, EMP3), consistent with infected erythrocyte rigidity and cytoadhesion (32, 37, 38) being important determinants of microvascular obstruction and hyperlactatemia (39). Pathway enrichments amongst these genes differed from those most associated with BCS and included membrane docking and rRNA processing (Fig 2D and table S6). Few parasite genes were associated with hemoglobin concentration or platelet count (table S7). Taken together, these findings indicate that different patterns of parasite gene expression are associated with, and may therefore contribute to, specific aspects of host pathophysiology.

In order to establish whether changes in parasite gene expression might be cause or consequence of severe malaria, we tested the effect of hyperlactatemia on parasite gene expression in vitro. 61 genes were differentially expressed between lactate supplemented (n = 4) and control (n = 5) early ring-stage parasite cultures, particularly enriched in genes associated with transcription and RNA processing (tables S5 and S6). Two of the genes most highly induced by lactate supplementation were PF3D7_1016300 (GBP) and PF3D7_0202000 (knob-associated histidine rich protein), genes which were also highly expressed in the cerebral malaria plus hyperlactatemia phenotype. This suggests lactate may influence the virulence phenotype of parasites, consistent with a recent report that Plasmodium can sense and respond to the host metabolic environment (40).

Previous studies have shown a correlation between the expression levels of host genes and circulating parasitemia (41, 42). Parasite load differed between our subjects with severe and uncomplicated malaria (Table 1) and we were interested to determine the extent to which this explained the differences in whole blood gene expression. Peripheral blood parasite quantification (parasite density) underestimates the total number of parasites in the body because of sequestration of parasites in the microvasculature (13, 14). The soluble parasite protein, P. falciparum histidine rich protein 2 (PfHRP2), has been used as a plasma biomarker of total parasite load (circulating plus sequestered parasites) and is more strongly associated with severity (8, 9, 11) and death (8, 9). We examined the association of host and parasite gene expression with both circulating parasite density and PfHRP2 (restricting comparisons to subjects with data for both). We...
found 1886 human genes significantly (FDR $P < 0.05$) correlated with log parasite density and 616
significantly correlated with log PfHRP2 (102 common to both), whilst only 2 and 10 parasite genes
were significant in the corresponding analyses (none common to both) (tables S4 and S7). Human
genes correlated with log parasite density were particularly enriched in pathways related to
translation (especially exported proteins), oxidative phosphorylation, and antigen presentation (Fig
2F, table S2), with predicted upstream regulation by RPTOR independent companion of MTOR
complex 2 (RICTOR), hepatocyte nuclear factor 4 alpha (HNF4A) and X-box binding protein 1 (XBP1);
table S3). Genes correlated with log PfHRP2 were enriched in innate immune response functions (Fig
2F, table S2), and the most strongly predicted upstream regulators were interferon-γ (IFN-γ),
transglutaminase 2, and IFN-α2 (table S3). These findings suggest that the nature of the systemic
host response is associated with the localisation of parasites.

We next asked to what extent the differences in gene expression between severe and
uncomplicated malaria phenotypes were dependent on parasite load. Restricting analyses to
subjects with both parasite density and PfHRP2 measurements, the number of human genes
differentially expressed in severe vs uncomplicated malaria remained almost unchanged after
adjustment for parasite density but was reduced by 98.6% after adjustment for PfHRP2, whilst
parasite differentially expressed genes changed much less after either of the same adjustments
(Table 2, tables S1 and S5). Findings were similar when adjusting for parasite load in comparisons of
each of the severe malaria subtypes vs uncomplicated malaria (tables S1 and S5). Repeating this
analysis on an independent dataset of human microarray gene expression in Malawian children with
cerebral malaria (43) revealed 994 differentially expressed genes (FDR $P < 0.05$) between children
with ($n = 55$) and without ($n = 17$) malaria-associated retinopathy (table S1), with 608 (61%) genes
found to be differentially expressed after adjustment for parasite density, and none differentially
expressed after adjustment for PfHRP2.

Taken together these findings suggest that total body parasite load, as represented by PfHRP2, is a
dominant determinant of host gene expression in malaria, particularly of inflammatory and immune
response genes, and differences in total body parasite load drive the majority of the human gene expression differences between severe and uncomplicated malaria. However, if genes remain associated with severity after adjustment for parasite load, this may indicate intrinsic variation in the host response which determines susceptibility to severe disease. In our dataset, only 13 genes remained significant (FDR $P < 0.05$) after adjustment for PfHRP2 (Table 2, table S1). Of particular interest amongst these, \textit{MMP8} encodes the metallopeptidase MMP8 (also known as collagenase 1) which causes endothelial barrier damage in several infection models \cite{44, 45}; \textit{AZI2} encodes 5-azacytidine induced 2 (also known as NF-Kappa-B-Activating Kinase-Associated Protein 1, NAP1) \cite{46}, a regulator of the type 1 interferon response, a pathway which is known to control severity of disease in rodent malaria models \cite{47}; whilst \textit{CX3CR1} encodes the receptor for fractalkine (a biomarker of cerebral malaria in humans \cite{48}), expressed on subset of monocytes which are particularly efficient at killing malaria parasites \cite{49}, and controls the trafficking of monocytes during inflammation \cite{50}.

Parasite load was also a major driver of the associations between human gene expression and BCS level, lactate, and platelet count, although platelet count-associated genes were relatively less dependent on parasite load (Table 2). The few remaining genes significantly associated with lactate after adjustment for PfHRP2 included \textit{PKM} (encoding the glycolytic enzyme pyruvate kinase M) and \textit{GYS1} (encoding the glycogenic enzyme glycogen synthase 1) (table S4), suggesting hyperlactatemia is partly associated with parasite load-independent variation in control of host glucose metabolism.

**Co-expression networks of host and parasite genes**

The expression of genes with common functional roles is often correlated and can be identified through co-expression network analysis \cite{51}. We applied this methodology to paired host and parasite gene expression data from each individual (without prior adjustment for parasite load) to identify co-expressed groups of genes from either or both species. We term these groups of genes “modules”, and we named each module according to the “hub gene” which has the greatest
connectivity to other genes within the module. First we analysed all subjects together, generating a network with 26 modules (Fig 3 and table S8): 10 modules contained exclusively human genes, 5 exclusively parasite genes, and 11 both human and parasite genes (most of these highly skewed to a single species). Only the HSPH1 (heat shock protein family H [Hsp110] member 1) module contained more than 10 genes from both human and parasite, and was strongly enriched in human heat shock response and parasite RNA metabolism genes. All modules showed significant ($P < 0.05$) GO enrichments, regardless of host or parasite origin. The composite expression of genes within a module can be described by a module eigengene value ($s_1, s_2$) and there were associations between module eigengene values and malaria severity, parasite load, consciousness level, and other laboratory parameters (Figure 3). Some host-dominated and parasite-dominated modules were also highly correlated with each other, most notably the RPL24 (ribosomal protein L24) module (highly enriched in translation pathways) strongly correlated with the functionally similar PF3D7_0721600 (putative 40S ribosomal protein 5S) parasite module. We excluded multi-mapping reads as an explanation for this, and suggest that this indicates co-regulation of conserved host and parasite translation machinery. Furthermore, most of these genes were also differentially expressed between severe and uncomplicated malaria.

**Association of co-expression modules with severity**

Co-expression network modules can be used as units of analysis, affording considerable dimension reduction for whole-genome expression data. We used module eigengene values and parasite load (with which many modules were correlated, Fig 3) in linear regression models to determine the best within-sample predictors of severity, starting with all significant ($P < 0.01$) univariate associations and proceeding by backward selection (table S9). The resulting multivariate model combined MMP8, OAS1 (2'-5'-oligoadenylate synthetase 1) and LYSMD3 (LysM, putative peptidoglycan-binding, domain containing 3) module eigengenes, but not parasite load. In fact, these modules represent distinct aspects of the immune response (table S8): the MMP8 module, highly enriched in defence
response genes with predicted upstream regulators CEBPA (CCAAT/enhancer binding protein alpha, a myeloid transcription factor) and CSF3, likely reflects granulopoiesis (31); the OAS1 module is highly enriched for type-1 IFN response genes; and the small LYSMD3 module, with limited GO enrichment, contains a functional network around IFN-γ (figure S4). The direction of association of the OAS1 module with severity changed from negative in univariate analysis to positive in the multivariate analysis, suggesting that inadequate suppression of the type-1 IFN response in conjunction with upregulation of granulopoiesis and IFN-γ signalling may contribute to pathogenesis.

**Differential co-expression in severe malaria**

Considering all subjects together in the generation of co-expression networks maximises power to detect consistently co-regulated genes but may not identify sets of genes where co-regulation is altered by severity. For this reason we also created separate co-expression networks for uncomplicated and severe malaria and compared modules to identify differential co-expression (Fig 4, table S10). Eight modules showed substantial preservation between networks, seven were partially preserved, and two were unique to severe malaria (Figure 4A). Partial preservation was common amongst modules comprised predominantly from human or parasite genes (Figure 4A,B), and module preservation was not dependent on the proportion of module genes differentially expressed between severe and uncomplicated malaria (Figure 4A,C). A MMP8 module (exclusively human genes, many encoding neutrophil granule and phagosome components) was uniquely present in severe malaria subjects, and 38% of its member genes were differentially expressed in the comparison between severe malaria and uncomplicated malaria (table S10). The module was enriched in host defence functions and predicted to be regulated by CEBPA, CSF3 and TNF (table S10). These findings strongly suggest the MMP8 module represents emergency granulopoiesis (31) and mark this as a specific feature of severe malaria. The TIPRL (TOR Signaling Pathway Regulator) module (99.2% human genes) was also unique to severe malaria but contained very few (1.3%) differentially expressed genes, had limited GO enrichment, and the most strongly predicted
upstream regulator was the transcription factor HNF4A (table S10). Both TIPRL and HNF4A have regulatory roles in metabolic, inflammatory and apoptosis signal pathways, so the minimal change in expression of this module may represent an aberrant response in severe malaria (53, 54). Amongst the partially preserved modules we observed that host and parasite translation pathways were more tightly co-regulated in severe than uncomplicated malaria, with genes being distributed across fewer modules in severe malaria (Fig 4A, table S10). This once again suggests that there is an interaction between these processes that is associated with severity.

Interaction of parasite and host gene expression accounting for parasite load

We sought to integrate pathogen load into analysis of interaction between host and parasite gene expression. To reduce dimensionality we generated human-only gene expression modules from all subjects (table S11), identified those modules significantly \( P < 0.05 \) associated with severity, and then identified parasite genes with significant (FDR \( P < 0.05 \)) pairwise associations with these modules in a linear model accounting for log PfHRP2. The human modules associated with severity were similar to those identified in preceding analyses (Table 3). The most significantly associated parasite genes and the most enriched parasite GO terms were those involved in RNA processing and translation (Table 3, table S11), suggesting that these processes in the parasite drive multiple aspects of the host transcriptional response independent of their effect on parasite load.

Neutrophil-related proteins in plasma

The most differentially expressed genes in comparisons between severe and uncomplicated malaria encode neutrophil granule proteins (figure 2A). Relationships between transcription, translation, storage and release of granule proteins are expected to be complex, but we sought evidence of correlation between gene expression and circulating protein concentrations. In subjects with residual stored plasma we found significant correlations between gene expression and plasma concentrations of defensin A3 \( P = 0.0049, \rho = 0.47, n = 34 \) and elastase \( P = 0.045, \rho = 0.35, n = \)
MMP8 expression was significantly correlated with plasma concentrations in subjects with severe malaria ($P = 0.02$, rho = 0.59, $n = 15$), but not in uncomplicated malaria ($P = 0.37$, rho = -0.21, $n = 20$) or all subjects combined ($P = 0.88$, rho = -0.026, $n = 35$). Upstream regulator analyses described earlier suggested that GCSF (CSF3) was a major regulator of genes in the MMP8 module. We found that plasma GCSF concentrations significantly correlated with the eigengene values (table S8) for this module ($P = 0.0030$, rho = 0.64, $n = 19$). We tested whether neutrophil degranulation occurred in response to parasite material by stimulating healthy donor blood cells with *P. falciparum* schizont lysate, and detected increases in MMP8 release, reaching similar concentrations to those observed in plasma during malaria (figure S5).

**Discussion**

We used dual RNA-sequencing to identify simultaneous host and parasite gene expression and their systemic interactions associated with severity of *P. falciparum* malaria in humans. Whilst gene expression is only one of the many biological processes involved, our findings add to the argument for an integrated understanding of infectious diseases and make a strong case that neither host nor pathogen should be studied in isolation when possible. We have identified many associations between gene expression and features of severity, providing plausible insight into the pathogenesis of severe malaria (figure S6). One of our most striking findings was the overriding effect of parasite load on differences in human gene expression between severe and uncomplicated malaria. Previous studies have examined the association between human gene expression and circulating parasitemia (23, 41, 42), but we found that estimation of total body (both circulating and sequestered) parasite load was necessary to appreciate the full effect on host response. Our findings imply that the host response in severe malaria is not excessive *per se*, but rather that it is an appropriate host response to an excessive pathogen load. This has important implications for malaria research and likely for other infectious disease, immunology, and pathogenesis research in humans. Without accounting for pathogen load, associations between host
factors (such as genetic variants or comorbidities) and severity of infection may be misinterpreted. Unfortunately, total body pathogen load is much harder to measure in other infections in humans where pathogens are not restricted to the blood (55). We found that specific sets of host and parasite genes were associated with different pathophysiological consequences of malaria, although our power to detect associations was limited in the smallest subgroup analyses. Distinct sets of host genes were associated with BCS, lactate concentration and platelet count. Hyperlactatemia in malaria is often ascribed to anaerobic metabolism arising from microvascular obstruction by adherent and rigid parasitized erythrocytes (4, 39). These properties are partly determined by the expression of particular members of the var, rif and stevor gene families (14), which we did not include in our analysis because their extreme degree of polymorphism prevents reference genome-based quantification. Despite this limitation we still found associations between lactate concentration and severity and in vivo variation in the expression of other parasite genes known to modify the biophysical properties of the infected erythrocyte. Some variation in parasite gene expression may have a genetic basis (56) but our in vitro data suggests that it may also occur as a response to the host environment.

The human genes most correlated with lactate were immune response-related, suggesting that inflammation, and perhaps its effect on glycolysis, may be involved (57, 58). If lactate production is associated with the strength of the host response then the changes in expression of parasite genes in response to lactate might favour sequestration and evasion of innate immune cells as a parasite survival strategy. Cerebral malaria in humans is usually ascribed to parasite sequestration in the cerebral microvasculature, but the association between BCS and antigen presentation via MHC class I, interferon-γ, and type-1 interferon signalling, would be consistent with the localisation of these immunopathological mechanisms away from the blood and into the brain microvasculature as seen in rodent experimental cerebral malaria (15, 16, 59). Thrombocytopenia is almost invariable in malaria and its mechanism is poorly understood (7). Our findings implicate the well-recognised
activation of endothelial surfaces and coagulation pathways in malaria (7) as a cause, but also lead
us to suspect a role for histone-induced thrombocytopenia (60, 61).

Many of our results converge on a putative role for neutrophils in severe malaria. Different analytical
approaches repeatedly identified the association of genes encoding neutrophil granules (such as
MMP8, OLFM4, DEFA3, ELANE) and upstream regulators of granulopoiesis (CSF3 and CEBPA) with
severe outcomes. Granule proteins are enriched in immature neutrophils (31, 62) which are
mobilised from the bone marrow to the circulation in malaria (63, 64), and there is plentiful
evidence that neutrophil degranulation occurs in severe malaria (43, 65). Release of neutrophil
granule proteins can be highly damaging to host tissues (62), and increased production and release
of these proteins could contribute to many of the pathological features of severe malaria. Both
neutrophil granule proteins and histones are released into the circulation during production of
neutrophil extracellular traps (NETs) (62, 66), a phenomenon which has been described in malaria
(64). It is noteworthy that similar neutrophil-related signatures are not reported in the whole blood
transcriptomes of rodent models which have been examined to date (42, 67), creating a challenge
for experimental validation. However, neutrophil depletion has been shown to prevent experimental
cerebral malaria (68), and whilst this is not a viable therapeutic option in humans, pharmacological
inhibitors of specific neutrophil functions such as NETosis are being evaluated in other diseases (66).

We observed a relationship between type-1 IFN responses and severity of malaria, which may help
to tie together data from previous observations in humans and animal models. In a small study,
expression of type-1 IFN response genes in blood from uncomplicated malaria was higher than in
severe malaria, leading to the suggestion that this may be protective against developing severe
malaria (69). However, we found that type-1 IFN response genes were negatively correlated with
parasite load, suggesting down-regulation with increasing parasite load (and severity) is a more likely
explanation. Our multivariate analyses using gene expression modules to explain severity suggested
that insufficient suppression of type-1 IFN signalling was in fact associated with severity. This would
be more consistent with results in mouse malaria models where genetic or antibody-mediated
ablation of type-1 IFN signalling improves outcome (70-73).

Very few parasite genes correlated with parasite load at the time of clinical presentation. This may
be a consequence of the dynamic nature of parasite load, which is determined by parasite growth
rate, the number of replication cycles in the host (duration of infection), and a reciprocal interaction
with the constraining host response. Thus lower expression of the gene encoding ApiAP2-G in severe
malaria may increase the asexual parasite growth rate (34) and make severe malaria more likely,
without this gene exhibiting any correlation with parasite load. It may also seem paradoxical that
this gene is down-regulated in severe malaria given that gametocytes are usually more common in
severe malaria (30). Development of mature gametocytes takes 10-12 days, for much of which they
are not in the systemic circulation (74). We speculate that the mature gametocytes detected at the
time of clinical presentation may reflect preferential gametocytogenesis in early infection and
perhaps a subsequent reduction in ApiAP2-G that promotes enhanced asexual replication and severe
disease.

We noted that both human and parasite translation pathways were associated with severe malaria,
and these pathways showed the strongest evidence of interaction between species, with co-
regulation appearing tighter in more severe disease. Increased translation is important for
production of host defence effector proteins (75) and parasite proteins which enable survival (76)
and it is feasible that these processes drive each other. This raises the intriguing question of whether
addition of a translation inhibiting anti-malarial such as mefloquine (77) to standard artesunate
treatment may have added benefit in severe malaria.

The differences in human and parasite gene expression between severe and uncomplicated malaria
were much clearer after adjusting for heterogeneity of leukocyte population and parasite
developmental stage. Although the importance of accounting for such variation is well recognised
(24), it is rarely done in infectious disease transcriptomic studies. Several studies have used
alternative methods to account for parasite developmental stage distribution and have shown that
this has a major impact on observed associations between parasite gene expression and clinical phenotype (78, 79).

Whilst we cannot establish causation from an observational study such as this, our findings should be launch points for future work assessing the implicated mechanisms and their potential as targets for adjunctive therapies. The identification of multiple and sometimes distinct host and parasite mechanisms associated with differing aspects of pathophysiology potentially bodes ill for adjunctive therapies, which might need to have multiple targets and perhaps be personalized to differing severe malaria manifestations. However the common association of neutrophil granule protein genes with all severe malaria manifestations suggests that targeting neutrophil function may be a therapeutic strategy in the future.
Materials and Methods

Detailed Supplementary Methods are available on-line

Study design

The primary aim of the study was to analyse differential human and parasite gene expression between children with severe malaria and uncomplicated malaria and to determine association of gene expression with parasite load. Secondary aims were 1) to analyse differences in gene expression associated with different severe malaria syndromes and with continuous variable markers of pathophysiology, 2) to evaluate co-expression of host and parasite genes, and 3) to evaluate differential co-expression associated with severe malaria. Sample size was determined pragmatically based on the availability of suitable samples with necessary clinical and laboratory data. We aimed to achieve close to 30 million mapped human reads and 5 million mapped parasite reads per sample and calculated the likely number of reads required to achieve this based on the percentage parasitemia in each subject and the likely amount of RNA per ring-stage parasite (27).

From available RNA samples we aimed to have roughly equal numbers of severe and uncomplicated malaria cases and within the severe malaria cases we aimed to have eight subjects with each phenotype. After assessment of RNA quantity and quality some samples were unsuitable for RNA-sequencing which resulted in the final composition of groups being slightly unbalanced.

Subjects and samples

Gambian children (under 16 years old) with *P. falciparum* malaria were recruited as previously described (11, 80, 81). Informed consent was obtained and the study was approved by the Gambian Government / MRC Laboratories Joint Ethics Committee (SCCs 670, 1077, 1143, 1178, 1179, 1180, 1207 and L2013.07V2). Malaria was defined by fever and >5000 asexual parasites/μL of blood. Cerebral malaria was defined as BCS of 1 or 2, or a BCS of 3 if the motor response was 1, not due to other causes (11, 80). Hyperlactatemia / acidosis was defined as blood lactate concentration > 5mmol/L (11). Subjects meeting both criteria were described as having cerebral malaria plus
hyperlactatemia / acidosis. Blood was collected at the time of presentation to the clinic, prior to any
treatment (80).

For RNA-sequencing, severe and uncomplicated malaria groups were matched as closely as possible
by age and gender (Supplementary Dataset 1). For uncomplicated malaria samples we aimed to
include an equal number with parasitemia above and below 5%.

RNA-sequencing

RNA was collected and extracted as described previously (80). 1μg of total RNA was used with the
ScriptSeq v2 RNA-sequencing library preparation kit (Illumina) and ribosomal RNA (rRNA) and globin
messenger RNA (mRNA) were depleted using the Globin-Zero Gold kit (Epicentre). Strand-specific
libraries were sequenced using the 2x100 bp protocol with an Illumina HiSeq 2500.

Genomes and RNA annotations

Reference genomes were obtained for human (hg38) (82) and P. falciparum (release 24) (83), and
gene annotations were obtained from GENCODE (release 22) (84) and PlasmoDB (release 24) (83).

Statistical analysis

Characteristics were compared between subject groups using the Kruskal-Wallis or Mann-Whitney
tests for continuous data and Fisher’s exact test for categorical data. The Wilcoxon matched pairs
test was used to compare paired data. Correlations were performed using Spearman correlation or
Pearson correlation (when data was normally distributed). To determine genome-wide gene
expression associated with variables of interest, P-values were calculated for individual genes as
described in the Supplementary Methods, and a false-discovery rate adjusted P < 0.05 was
considered significant. Fisher’s exact test was used to identify gene set enrichments. Logistic
regression was used to determine associations between severity and module eigengene values.
Supplementary Materials

Supplementary Materials and Methods

Fig S1. Estimates of the relative proportions of leukocyte subpopulations in subjects with severe and uncomplicated malaria.

Fig S2. Validation of the gene-signature approach to estimate parasite developmental stage proportions.

Fig S3. Differential gene expression between severe malaria phenotypes and uncomplicated malaria.

Fig S4. Top functional network for the small LYSMD3 module.

Fig S5. Association between gene expression and plasma protein concentrations.

Fig S6. Host pathogen-interactions in severe malaria revealed through dual RNA-sequencing.

Table S1. Human genes differentially expressed between malaria disease phenotypes in unadjusted and parasite load-adjusted analyses.

Table S2. Gene ontology terms associated with human differentially expressed or significantly correlated genes in unadjusted and parasite load-adjusted analyses.
Table S3. Predicted upstream regulators associated with human differentially expressed or significantly correlated genes in unadjusted and parasite load-adjusted analyses.

Table S4. Human genes significantly correlated with parasite load and pathophysiological variables in unadjusted and parasite load-adjusted analyses.

Table S5. *P. falciparum* genes differentially expressed in unadjusted and parasite load-adjusted analyses.

Table S6. Gene ontology terms associated with parasite differentially expressed or significantly correlated genes in unadjusted and parasite load-adjusted analyses.

Table S7. *P. falciparum* genes significantly correlated with parasite load and pathophysiological variables in unadjusted and parasite load-adjusted analyses.

Table S8. Summary of modules obtained from combined whole genome correlation network.

Table S9. Univariate and multivariate associations of module eigengene values and parasite load with severity.

Table S10. Summary and overlap of whole genome correlation sub-networks for severe and uncomplicated malaria.

Table S11. Summary of modules obtained from human only whole genome correlation network.

Supplementary Dataset 1. Subject-level clinical and laboratory data.
References and Notes


44. R. E. Vandenbroucke, E. Dejonckheere, P. Van Lint, D. Demeestere, E. Van Wonterghem, I.
   Lopez-Otin, P. Matthys, G. Leclercq, C. Libert, Matrix metalloprotease 8-dependent
   extracellular matrix cleavage at the blood-CSF barrier contributes to lethality during systemic

45. A. Schubert-Unkmeir, C. Konrad, H. Slanina, F. Czapek, S. Hebling, M. Frosch, Neisseria
   meningitidis induces brain microvascular endothelial cell detachment from the matrix and

46. M. Sasai, H. Oshiumi, M. Matsumoto, N. Inoue, F. Fujita, M. Nakanishi, T. Seya, Cutting Edge:
   NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology
   domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. *J


48. R. Tahar, C. Albergaria, N. Zeghidour, V. F. Ngane, L. K. Basco, C. Roussilhon, Plasma levels of
   eight different mediators and their potential as biomarkers of various clinical malaria

49. P. Chimma, C. Roussilhon, P. Sratongno, R. Ruangveerayuth, K. Pattanapanyasat, J. L.
   Perignon, D. J. Roberts, P. Druilhe, A distinct peripheral blood monocyte phenotype is
   associated with parasite inhibitory activity in acute uncomplicated Plasmodium falciparum

50. P. Hamon, P. L. Loyher, C. Baudesson de Chanville, F. Licata, C. Combadiere, A. Boissonnas,
   CX3CR1-dependent endothelial margination modulates Ly6C(high) monocyte systemic


853 89. M. D. Robinson, D. J. McCarthy, G. K. Smyth, edgeR: a Bioconductor package for differential

854 90. A. R. Abbas, D. Baldwin, Y. Ma, W. Ouyang, A. Gurney, F. Martin, S. Fong, M. van Lookeren
Campagne, P. Godowski, P. M. Williams, A. C. Chan, H. F. Clark, Immune response in silico
(IRIS): immune-specific genes identified from a compendium of microarray expression data.


856 91. Y. Benjamini, Y. Hochberg, Controlling false discovery rate: a practical and powerful

857 92. F. Supek, M. Bosnjak, N. Skunca, T. Smuc, REVIGO summarizes and visualizes long lists of

simple-to-use R package for the circular visualization of multidimensional omics data. *Cancer
Acknowledgements: We are grateful to the study subjects, staff at MRCG, Jammeh Foundation for Peace Hospital, and Brikama Health Centre; Konrad Paszkiewicz and staff at Exeter Sequencing Service at the University of Exeter; Johanna Daily for providing subject level data for GSE72058.

Funding: This work was funded by the UK Medical Research Council (MRC) and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement and is also part of the EDCTP2 programme supported by the European Union (MR/L006529/1 to AJC), the European Research Council (AdG-2011-294428 to DJC and LBS), MRC core funding of the MRC Gambia Unit (MRCG), and the Wellcome Trust (098051 to AJC); Exeter Sequencing Service is supported by Medical Research Council Clinical Infrastructure award (MR/M008924/1), Wellcome Trust Institutional Strategic Support Fund (WT097835MF), Wellcome Trust Multi User Equipment Award (WT101650MA) and BBSRC LOLA award (BB/K003240/1). Author contributions: AJC, ML and DJC conceived the study; HJL, TDO and AG performed formal analysis; AG, MW and AJC performed laboratory analyses; MW, DJC, DN and LBS provided samples; DJC, TDO and LBS provided methodology; AJC and HJL wrote the original draft; all authors contributed to review and editing of the manuscript; LJC, DJC, ML, TDO and AJC provided supervision; AJC obtained funding. Competing interests: The authors declare no competing financial interests. Data availability: RNA-seq data from human samples have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6413. The accession number for in vitro parasite RNA-seq is E-MTAB-6573. Source data for Table 1 are provided with the paper as Supplementary Dataset 1. Correspondence should be addressed to Dr Aubrey Cunnington, Clinical Senior Lecturer, Section of Paediatrics, Department of Medicine, Imperial College London (St Mary's Campus) 231, Medical School Building, Norfolk Place, London W2 1PG. a.cunnington@imperial.ac.uk
<table>
<thead>
<tr>
<th></th>
<th>CM (n = 5)</th>
<th>CH (n = 12)</th>
<th>HL (n = 8)</th>
<th>UM (n = 21)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>4.3 (4.2-4.8)</td>
<td>4.9 (3.6-5.7)</td>
<td>5.0 (3.8-8.3)</td>
<td>6.0 (4.0-9.0)</td>
<td>0.51</td>
</tr>
<tr>
<td>Male (%)</td>
<td>3 (60%)</td>
<td>5 (42%)</td>
<td>7 (88%)</td>
<td>13 (62%)</td>
<td>0.24</td>
</tr>
<tr>
<td>Parasitemia (%)</td>
<td>8.3 (5.3-9.0)</td>
<td>12.6 (9.4-19.0)</td>
<td>9.6 (1.8-12.2)</td>
<td>5.1 (3.8-7.0)</td>
<td>0.008</td>
</tr>
<tr>
<td>Parasites (x10^9/uL)</td>
<td>2.3 (1.7-3.1)</td>
<td>3.5 (2.7-8.4)</td>
<td>2.8 (0.7-5.0)</td>
<td>2.3 (1.6-3.2)</td>
<td>0.062</td>
</tr>
<tr>
<td>Clones</td>
<td>2 (1.5-2.5)</td>
<td>2 (1-2)</td>
<td>1 (1-2)</td>
<td>2 (1-2)</td>
<td>0.67</td>
</tr>
<tr>
<td>PfHRP2 (ng/mL)</td>
<td>202 (93-528)</td>
<td>763 (374-1750)</td>
<td>470 (164-2214)</td>
<td>163 (128-227)</td>
<td>0.004</td>
</tr>
<tr>
<td>Duration of illness (days)</td>
<td>2.0 (1.7-3.0)</td>
<td>2.0 (2.0-2.5)</td>
<td>2.0 (2.0-3.5)</td>
<td>2.7 (2.0-3.0)</td>
<td>0.22</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>9.7 (7.4-10.4)</td>
<td>9.3 (7.8-11.5)</td>
<td>9.1 (7.4-11.0)</td>
<td>10.8 (9.9-12.1)</td>
<td>0.12</td>
</tr>
<tr>
<td>WBC (x10^9/L)</td>
<td>9.8 (8.2-12.9)</td>
<td>8.8 (6.4-9.4)</td>
<td>15.3 (7.9-16.8)</td>
<td>9.5 (7.7-11.8)</td>
<td>0.41</td>
</tr>
<tr>
<td>Platelets (x10^9/L)</td>
<td>41 (40-82)</td>
<td>36 (23-65)</td>
<td>59 (33-132)</td>
<td>122 (96-132)</td>
<td>0.013</td>
</tr>
<tr>
<td>Lymphocytes (x10^9/L)</td>
<td>2.7 (2.1-3.6)</td>
<td>2.9 (2.4-3.6)</td>
<td>3.1 (1.8-5.2)</td>
<td>2.4 (1.4-3.1)</td>
<td>0.57</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>29.8 (20.6-37.3)</td>
<td>37.8 (29.9-49.9)</td>
<td>22.3 (14.7-37.3)</td>
<td>23.9 (16.0-33.5)</td>
<td>0.087</td>
</tr>
<tr>
<td>Neutrophils (x10^9/L)</td>
<td>6.4 (4.0-8.7)</td>
<td>4.0 (2.9-4.3)</td>
<td>6.5 (5.8-10.4)</td>
<td>7.0 (5.3-7.7)</td>
<td>0.045</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>55.1 (49.0-69.6)</td>
<td>48.3 (39.6-56.2)</td>
<td>61.5 (55.6-74.9)</td>
<td>68.0 (59.9-79.6)</td>
<td>0.016</td>
</tr>
<tr>
<td>Monocytes (x10^9/L)</td>
<td>0.6 (0.6-0.7)</td>
<td>0.6 (0.5-0.9)</td>
<td>0.8 (0.6-1.3)</td>
<td>0.7 (0.4-0.9)</td>
<td>0.58</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>7.1 (6.0-7.7)</td>
<td>7.8 (6.8-8.6)</td>
<td>6.6 (5.1-7.8)</td>
<td>6.7 (4.8-7.3)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of study subjects (n = 46). CM, cerebral malaria; CH, cerebral malaria plus hyperlactatemia; HL, hyperlactatemia (CM, CH, and HL are all subgroups of severe malaria); UM, uncomplicated malaria; PfHRP2, *P. falciparum* histidine rich protein 2; Hb, hemoglobin concentration; WBC, white blood cell count. Data are median (IQR), superscripts indicate the number of subjects with data for each variable if less than the total; *P* for Kruskall-Wallis test.
comparing all groups (degrees of freedom = 3) except for sex where $P$ is for Fisher’s exact test.

Leukocyte population numbers and proportions measured by clinical hematology analyser.
<table>
<thead>
<tr>
<th></th>
<th>Human genes</th>
<th></th>
<th>Parasite genes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log parasite density</td>
<td>Log PfHRP2</td>
<td>n* Unadjusted</td>
<td>Log parasite density</td>
</tr>
<tr>
<td>SM vs UM</td>
<td>43</td>
<td>907</td>
<td>914</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>(101%)</td>
<td>(1.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCS</td>
<td>43</td>
<td>738</td>
<td>491</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>(67%)</td>
<td>(1.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>40</td>
<td>1012</td>
<td>526</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>(52%)</td>
<td>(5.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td>43</td>
<td>178</td>
<td>66</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>(37%)</td>
<td>(25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>43</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 2. Numbers of differentially expressed genes before and after adjustment for parasite load.

*Only subjects with complete data for every parameter are included. Number of genes associated with severity category or laboratory marker of severity before and after adjustment for parasite load (% of number in unadjusted analysis where applicable). BCS, Blantyre coma scale; SM, severe malaria; UM, uncomplicated malaria.
<table>
<thead>
<tr>
<th>Human Module Correlated parasite genes after adjustment for parasite load</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hub gene</strong></td>
</tr>
<tr>
<td>Receptor Transporter Protein 4 (RTP4)</td>
</tr>
<tr>
<td>Trinucleotide Repeat Containing 6B (TNRC6B)</td>
</tr>
<tr>
<td>Heat shock protein family H (Hsp110) member 1 (HSPH1)</td>
</tr>
<tr>
<td>Matrix metallopeptidase 8 (MMP8)</td>
</tr>
<tr>
<td>GO:0034976 response to endoplasmic reticulum stress</td>
</tr>
<tr>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>PF3D7_1420300 (DNL-type zinc finger protein)</td>
</tr>
<tr>
<td>PF3D7_0821200 (unknown function)</td>
</tr>
<tr>
<td>PF3D7_1119200 (unknown function)</td>
</tr>
</tbody>
</table>

**Table 3.** Parasite genes correlated with human gene co-expression modules after adjustment for parasite load. +/- indicates number of parasite genes positively / negatively correlated with each human module eigengene value. All “top genes” in the table are positively correlated with the module eigengene value.
Figure 1. Whole blood dual RNA-sequencing and deconvolution. (A) Uniquely mapped reads from human (red) and *P. falciparum* (blue) from subjects with severe (SM, *n* = 25) and uncomplicated malaria (UM, *n* = 21). (B, C) Heatmaps showing signature gene expression for different leukocyte (B) and parasite developmental stage (C) populations and their relative intensity in individual subjects with SM, including different SM phenotypes (CH, cerebral malaria plus hyperlactatemia; CM,
cerebral malaria; HL, hyperlactatemia), and UM. (D) Surrogate proportion variables for parasite developmental stages compared between severe malaria and uncomplicated malaria using the Mann-Whitney test (bold line, box and whiskers indicate median, interquartile range and up to 1.5-times interquartile range from the lower and upper ends of the box respectively). (E,F) Principal component plots showing the effect of deconvolution on the segregation of subjects with UM and SM, adjusting human (E) and parasite (F) gene expression for differences in proportions of leukocytes or parasite developmental stages respectively. Analyses of human gene expression (B,E): SM, n = 25; UM, n = 21. Analyses of parasite gene expression (C,D,F): SM, n = 23; UM, n = 20.
Figure 2. Association of gene expression with features of severe malaria and parasite load. (A)

Volcano plot showing extent and significance of up- or down-regulation of human gene expression in severe malaria (SM) compared with uncomplicated malaria (UM) (red and blue, P < 0.05 after...
Benjamini-Hochberg adjustment for false discovery rate [FDR]; orange and blue, absolute log₂-fold change (FC) in expression > 1; SM \( n = 25 \), UM \( n = 21 \). (B) Comparison of selected neutrophil-related gene expression multiplied by absolute neutrophil count in blood between SM \( n = 21 \) and UM \( n = 20 \) (bold line, box and whiskers indicate median, interquartile range and up to 1.5-times interquartile range from the lower and upper ends of the box respectively, \( P \) for Mann-Whitney test). (C) Heatmap comparing enrichment of gene ontology terms for human genes significantly differentially expressed between severe malaria and uncomplicated malaria or significantly associated with blood lactate, platelet count or BCS. (D) \( P. falciparum \) differential gene expression in severe malaria compared to uncomplicated malaria (colour coding as in (A); SM \( n = 23 \), UM \( n = 20 \)). (E) Heatmap comparing enrichment of gene ontology terms for parasite genes significantly differentially expressed between severe malaria and uncomplicated malaria and or significantly associated with blood lactate or BCS. (F) Heatmap comparing gene ontology terms for human genes significantly associated with log parasite density and log PfHRP2.
Figure 3. Interspecies gene expression modules and their associations with severity. Circos plot showing gene expression modules obtained from whole genome correlation network analysis using
expression of all human and parasite genes from each subject (severe malaria, $n = 22$; uncomplicated malaria, $n = 19$) as the input. From outside to inside: labels, hub gene and most enriched GO term (with $P$-value) for each module; track 1, module eigengene value for each subject; track 2, clinical phenotype (Red, CH; Orange, CM; Green, HL; Yellow, uncomplicated malaria); track 3, hub gene expression (log CPM) for each subject; track 4, heatmap for correlation with laboratory measurements (clockwise, blocks: log parasite density, log PfHRP2, lactate, platelets, hemoglobin, BCS; colour intensity represents correlation coefficient as shown in colour key); track 5, module size and composition (length proportional to number of genes in module; red, human genes; blue, parasite genes); polygons connect modules with significant (FDR $P < 0.01$) Pearson correlation between eigengene values (width proportional to $-\log_{10}$ FDR $P$-value; red=positive correlation, blue=negative correlation).
Figure 4. Severity-associated differential co-expression within the interspecies gene expression network. (A-C) Cytoscape visualisation of merged co-expression networks derived separately from severe malaria (n = 22) and uncomplicated malaria (n = 19). Networks were merged such that genes found in both sub-networks (represented as arrow-shaped, larger-sized nodes) are connected to
genes found in only one sub-network (represented as circular-shaped and smaller-sized nodes). (A)

Genes and gene clusters are coloured and annotated by module, species, most enriched gene ontology terms, and conservation between sub-networks. Preserved, module pairs from severe malaria and uncomplicated malaria sub-networks overlap with each other but no other modules; partially preserved, module clusters in one sub-network overlap with two or more modules in the other sub-network; unique, gene clustering only found in one sub-network. Genes in black do not belong to any characterized module. (B) Identical network layout with genes coloured by species (red, human; blue, P. falciparum). (C) Identical network layout with genes coloured by whether they are significantly differentially expressed in severe malaria vs uncomplicated malaria (red, human; blue, P. falciparum; black, not differentially expressed).