UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ – FIOCRUZ
CENTRO DE PESQUISAS GONÇALO MONIZ

Curso de Pós-Graduação em Patologia

TESE DE DOUTORADO

ASSOCIAÇÃO ENTRE BIOMARCADORES IMUNOGENÉTICOS E GRAVIDADE OU RESISTÊNCIA NA MALÁRIA POR *PLASMODIUM VIVAX*

VITOR ROSA RAMOS DE MENDONÇA

Salvador – Bahia

2015

UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ – FIOCRUZ
CENTRO DE PESQUISAS GONÇALO MONIZ

Curso de Pós-Graduação em Patologia

ASSOCIAÇÃO ENTRE BIOMARCADORES IMUNOGENÉTICOS E GRAVIDADE OU RESISTÊNCIA NA MALÁRIA POR *PLASMODIUM VIVAX*

VITOR ROSA RAMOS DE MENDONÇA

Orientador: Manoel Barral-Netto

Tese apresentada ao Curso de Pós-graduação em Patologia para a obtenção do grau de Doutor

Salvador – Bahia

2015

A Deus, por me guiar pelos melhores caminhos;

Aos meus pais, Wagner e Vera, e meus

irmãos, Wagner Filho e Juliana, pelo

amor incondicional e apoio constante.

AGRADECIMENTOS

Ao meu orientador, Dr Manoel Barral-Netto, pelas inúmeras oportunidades disponibilizadas, pelos desafios impostos para o meu aperfeiçoamento científico, e por me ensinar a fazer Ciência de modo crítico, maduro e entusiástico;

À Dra Marilda Gonçalves, pela disponibilidade constante e ensinamentos da Genética;

Ao Dr Bruno Andrade, pelos conselhos, cobranças e aprendizados;

Ao Dr Marcus Lacerda e toda equipe do FMT-HVD em Manaus, pelo apoio durante minha experiência na área endêmica; colaboração no acesso aos materiais e dados de pacientes portadores da malária e orientação no seu cuidado;

À Dra Aldina Barral, por ter aberto as portas da FIOCRUZ quando eu ainda era um estudante iniciante de medicina, e por continuar acreditando no meu potencial por toda esta jornada;

Às alunas de iniciação científica, Ligia Correa e Gabriela Barreto, pela ajuda nos experimentos;

À Elze Leite, Andrezza Karinny, Natali Alexandrino e Jorge Tolentino pelo apoio logístico;

À Silvana Paz, por toda ajuda na Plataforma de Seqüenciamento do CPqGM/FIOCRUZ-BA;

A todos os colaboradores científicos dos manuscritos desta tese;

Aos amigos e colegas do LIMI/LIP;

Á Ana Maria Fiscina Vaz Sampaio, da Biblioteca do CPqGM, pela ajuda na formatação final desta tese;

A todos que de forma direta ou indireta contribuíram para este trabalho.

SUMÁRIO

MENDONCA, Vitor Rosa Ramos de. Associação entre biomarcadores imunogenéticos e a gravidade ou resistência na malária por *Plasmodium vivax*. 177 f. il. Tese (Doutorado) – Universiadede Federal da Bahia. Centro de Pesquisas Gonçalo Moniz, Salvador, Bahia, 2015.

RESUMO

INTRODUÇÃO: A malária é uma das doenças infecto-parasitárias mais incidentes no mundo com grande morbimortalidade. Dentre as espécies infectivas ao ser humano, o *Plasmodium vivax* é a espécie predominante no Brasil, quase que exclusivamente na Região Amazônica. O espectro clínico da malária abrange desde uma infecção assintomática até casos moderados, com hiperbilirrubinemia isolada ou graves. A produção de mediadores inflamatórios pelo sistema imune, a via de metabolização do heme e os níveis sistêmicos de hepcidina são importantes mecanismos associados a fisiopatologia dos diferentes desfechos clínicos da malária. Além disso, coinfecções podem modular ou intensificar a resposta imune de indivíduos infectados pelo plasmódio. OBJETIVO: Neste ínterim, a identificação de biomarcadores confiáveis tanto de gravidade ou resistência são indispensáveis para o auxílio no seguimento, diagnóstico e terapêutica da malária. MATERIAL E MÉTODOS: Esta Tese reúne sete artigos com o objetivo de identificar biomarcadores plasmáticos e genéticos associados com a via do heme, hepcidina e resposta imune em indivíduos com diferentes manifestações clínicas da malária por *P. vivax*, assim como nos casos de coinfecção com dengue*,* da Amazônia Brasileira. RESULTADOS: Em primeiro lugar, foi descrito o estado da arte da diversidade genética humana que influencia o desfecho clínico da malária. Em outro estudo, alterações genéticas específicas e níveis plasmáticos elevados em moléculas na via de metabolização do heme estão associados com a malária vivax aguda. Em terceiro estudo, a interação entre biomarcadores imunoinflamatórios foi analisada por redes, e os participantes com malária assintomática tiveram várias correlações significativas envolvendo a IL-4. A resposta imune também foi influenciada por alterações genéticas e, em outro trabalho, foram identificados polimorfismos em genes relacionados ao sistema imune (*DDX39B*, *TNF* e *IL6)* que estão associados com risco maior de complicações na malária vivax, provavelmente, por influenciarem os níveis de mediadores inflamatórios. Verificou-se no quinto manuscrito que a resposta imune de indivíduos com malária e hiperbilirrubinemia isolada é parecida com aquela de indivíduos com malária não-complicada, sugerindo uma doença menos grave. Neste mesmo estudo, os níveis de hepcidina estão aumentados nos casos graves ou malária com hiperbilirrubinemia e este hormônio está positivamente correlacionado com IL-6, IL-10 e parasitemia no grupo de malária moderada, e com IFN-γ nos indivíduos graves. No sexto trabalho, um algoritmo clínico conseguiu distinguir os indivíduos monoinfectados daqueles com coinfecção dengue e *P. vivax*, e o perfil imune dos casos coinfectados foi marcada por uma assinatura molecular envolvendo as citocinas TNF, IL-6 e IFN-γ. Por fim, é descrito o estado da arte dos fatores imunoreguladores humanos relacionados à malária assintomática. CONCLUSÕES: O conjunto dos dados desta Tese ajuda no entendimento dos mecanismos imunopatológicos na infecção pelo *P. vivax* e podem guiar e auxiliar estudos futuros sobre métodos diagnósticos, terapêuticas e vacinas na malária vivax.

Palavras-chave: Malária**,** *P. Vivax*, Biomarcadores, Heme, Sistema Imune.

MENDONCA, VITOR R. R. Association between immunogenetic biomarkers and severity or resistance to *Plasmodium vivax* malaria*.* 177 f. il*.* Tese (Doutorado) – Universiadede Federal da Bahia. Centro de Pesquisas Gonçalo Moniz, Salvador, Bahia, 2015.

ABSTRACT

INTRODUCTION: Malaria is one of the most frequent infectious diseases in the world with high morbidity and mortality. Among the infective species to humans, *Plasmodium vivax* is the most predominant species in Brazil, with disease incidence almost exclusively observed in the Amazon Region. The clinical spectrum of malaria can range from asymptomatic infection to mild cases, malaria with isolated hyperbilirubinaemia or severe infection. The immune system production of inflammatory mediators, the heme metabolism pathway and systemic levels of hepcidin are important mechanisms associated with pathophysiology of different malaria clinical outcomes. In addition, co-infections can modulate or enhance the immune response of individuals infected with *P. vivax*. OBJECTIVE: In this context, the identification of reliable biomarkers for disease severity and resistance are essential for the diagnosis, treatment and follow-up of malaria. MATERIAL AND METHODS: This work, composed of seven articles, aims to better explain genetic and plasma biomarkers associated with the heme pathway, hepcidin and immune response occurring in *P.vivax* infection. Subjects have been sampled from the Brazilian Amazon, with different clinical manifestations of *P. vivax* malaria, as well as co-infected with dengue. RESULTS: Firstly, it is described the state of the art regarding human genetic diversity, which affects malaria outcomes. In another study, specific genetic alterations and high levels of heme metabolism pathway biomarkers are associated with acute vivax malaria. In the third report, interactions between immunoinflammatory networks were analyzed, and subjects with asymptomatic malaria had significant correlations with increased IL-4 levels. The immune response is also influenced by genetic factors. Towards it, a study has identified polymorphisms in certain immune system genes (*DDX39B*, *TNF* and *IL-6*) that are associated with an increased risk of complications in vivax malaria infection. The fifth manuscript shows that the immune response of subjects with malaria with hyperbilirubinemia is similar to patients with uncomplicated malaria, suggesting it as a less severe disease. Next, a clinical algorithm was shown to distinguish between monoinfected subjects from those co-infected with dengue and *P. Vivax*; and an immune signature composed by TNF, IL-6 and IFN-γ was found in co-infected subjects. Finally, it is described new human immunoregulatory factors related to asymptomatic malaria. CONCLUSIONS: Together, the data presented here contribute to better understanding the immunopathological mechanisms occurring throughout *P. vivax* infection, in order to guide and assist future studies about diagnosis, therapeutics and vaccination for *P. vivax* malaria infection.

Keywords: Malaria, *P. Vivax*, Biomarkers, Heme, Immune System.

LISTA DOS ARTIGOS

Esta tese é baseada nos seguintes manuscritos, os quais serão referidos pelos seus numerais romanos:

Manuscrito I

The Host Genetic Diversity in Malaria Infection

Journal of Tropical Medicine, v. 2012, p. 1-17, 2012

Manuscrito II

Association between the *Haptoglobin* and *Heme Oxygenase-1* Genetic Profiles and Soluble CD163 in Susceptibility to and Severity of Human Malaria.

Infection and Immunity, v. 80, p. 1445-1454, 2012

Manuscrito III

Networking the host immune response in *Plasmodium vivax* malaria.

Malaria Journal, v. 12, p. 1-10, 2013.

Manuscrito IV

***DDX39B (BAT1)*, *TNF* and *IL6* gene polymorphisms and association with clinical outcomes of patients with *Plasmodium vivax* malaria.**

Malaria Journal, v. 13, p. 278, 2014.

Manuscrito V

Associations between Hepcidin and Immune Response in Individuals with Hyperbilirubinaemia and Severe Malaria due to *Plasmodium vivax* Infection.

Malaria Journal, v. 14, p. 407, 2015

Manuscrito VI

Unraveling the patterns of host immune responses in *Plasmodium vivax* malaria and dengue coinfection.

Malaria Journal, v. 14, p. 315, 2015

Manuscrito VII

Immunoregulation in human malaria: the challenge of understanding asymptomatic malaria.

Mem Inst Oswaldo Cruz, v. 110(8), p. 945-955, 2015

LISTA DE ABREVIATURAS

ACP	Análise do componente principal
ALT	Alanina aminotransferase
AST	Aspartato transaminase
BAT1	HLA-B-associada ao transcrito 1
CCL	Motivo C-C ligante
CD	Grupamento de diferenciação
CO	Monóxio de carbono
CXCL	Motivo C-X-C ligante
DDX39B	Caixa polipeptídica 39B DEAD [Asp-Glu-Ala-Asp]
DNA	Ácido desoxirribonucleico
EDTA	Ácido etilenodiamino tetra-acético
ELISA	Ensaio imunoadsorvente ligado à enzima
ERO	Espécies reativas de oxigênio
Fe	Ferro
FMT-HVD	Fundação de Medicina Tropical Dr Heitor Vieira Dourado
FUNASA	Fundação Nacional de Saúde
Hb	Hemoglobina
HIV	Virus da imunodeficiência humana
HLA	Complexo principal de histocompatibilidade
HMOX1	Heme oxigenase [decíclica] 1
HO-1	Heme oxigenase-1
Hp	Haptoglobina
IFN	Interferon
Ig	Imunoglobulina

IL	Interleucina
JNK	Quinase c-Jun N-terminal
OMS	Organização Mundial de Saúde
PAMPs	Padrões moleculares associados á patógenos
PCR	Reação em cadeia da polimerase
PGE2	Prostaglandina E2
RB	Redes Bayesianas
RN	Redes neurais
RNAm	Ácido ribonucléico mensageiro
ROC	Caracteristicas *de operação do* receptor
sCD163	CD163 solúvel
SLC40A1	Família de carreador solúvel 40 membro 1
SNP	Polimorfismo de nucleotídeo único
SOD-1	Superóxido dismutase-1
STAT	Tradutor de sinal e ativador de transcrição
TCR	Receptor de células T
TGF-β	Fator de transformação do crescimento-β
TLRs	Receptor tipo toll
TNF	Fator de necrose tumoral
Treg	Células T regulatórias

LISTA DE FIGURAS

1. INTRODUÇÃO

1.1 EPIDEMIOLOGIA

A malária é uma das doenças infecto-parasitárias mais importantes e incidentes no mundo. Em 2013, foram registrados 198 milhões de casos clínicos de malária que resultaram em 584.000 mortes (WHO, 2014). Na América Latina, 427.000 casos e 82 mortes pela malária foram descritos em 2013, e o Brasil foi responsável por 42% (177.767) e 50% (41) destas notificações e óbitos, respectivamente (WHO, 2014).

Os parasitas da malária são classificados no filo *Protozoa*, classe *Sporozoea*, família *Plasmodiidae*, gênero *Plasmodium*. Cinco espécies são responsáveis por praticamente todas as infecções humanas: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* e *P. knowlesi*. A transmissão natural da malária ocorre por meio da picada de fêmeas de mosquitos do gênero *Anopheles,* previamente infectados por algum enfermo ou portador assintomático, sendo mais importante a espécie *Anopheles darlingi*, cujos criadouros preferenciais são coleções de água limpa, quente, sombreada e de baixo fluxo, muito frequentes na Amazônia Brasileira (WHO, 2014).

A forma infectante inicial do parasita chama-se esporozoíta e penetra no organismo através da saliva que o mosquito introduz no sangue dos capilares subcutâneos. O esporozoíto invade os hepatócitos e após divisões múltiplas, formam-se esquizontes teciduais primários, que passado o prazo de maturação de 6 a 16 dias a partir da infecção, rompem-se e liberam milhares de merozoítos. Nas infecções pelo *P. falciparum* e *P. malariae,* todos os esquizontes teciduais rompem ao mesmo tempo. Entretanto, nas

infecções pelo *P. vivax* e *P. ovale* alguns esquizontes, que recebem a denominação de hipnozoítas, permanecem latentes no fígado por meses ou anos, e estão relacionados pelas recidivas tardias nas infecções por estas espécies (COGSWELL et al., 1983; KROTOSKI, 1985).

Os merozoítos liberados nos vasos hepáticos invadem os eritrócitos e transformam-se em trofozoítas jovens, conhecidos como formas em anel, que crescem e tornam-se irregulares (trofozoítas amebóides) e, após múltiplas divisões transformam-se em esquizontes hemáticos, que originam, posteriormente, os merozoítos hemáticos. Os merozoítos liberados na circulação voltam a infectar outros eritrócitos e repetem o ciclo. Uma pequena proporção dos merozoítos presente nos eritrócitos diferencia-se em gametócitos que serão ingeridos pela fêmea do *Anopheles*. Esses gametas fusionam e se transformam em oocistos, que se dividem assexuadamente em vários esporozoítos, os quais migram para as glândulas salivares, de onde serão liberados durante o próximo repasto sanguíneo.

1.2 MALÁRIA POR *Plasmodium vivax*

O *Plasmodium vivax* é geograficamente o parasito mais amplamente distribuído da malária humana, com cerca de 2,5 bilhões de pessoas sob risco de transmissão no mundo, sobretudo na América Latina, Sudeste e Região Central da Ásia (GETHING et al., 2012). A baixa transmissão da malária vivax na África pode ser consequência da taxa alta relacionada a ausência do grupo sanguíneo Duffy, que permite a invasão do *P. vivax* nos eritrócitos, nas populações africanas. No Brasil, até a década de 80, existiu uma relativa equivalência entre as espécies parasitárias (*P. vivax* e *P. falciparum*) inclusive com um período de inversão parasitária, de 1983 a

1988, com predominância de *P. falciparum* (MS, 2014). A partir de então, nota-se o distanciamento no número de registro das duas espécies, fato que culminou com a predominância do *P. vivax*, responsável por mais de 95% dos casos notificados em 2013 quase que exclusivamente na Região Amazônica Brasileira (MS, 2014).

Apesar desta grande carga de doença, o *P. vivax* é negligenciado e ficou a sombra do problema causado por *P. falciparum* na África Sub-Saariana, no que diz respeito à maior mortalidade, sobretudo em crianças (MENDIS et al., 2009). A carga total de malária nas Américas é baixa quando comparada com a África, mas o *P. vivax* é responsável por mais de 70% da malária nas Américas e localmente provoca uma clínica substancial e grande problema socioeconômico (WHO, 2014). Este descaso tem refletido no montante aplicado em pesquisas relacionadas à doença visto que, no período de 2006 a 2009, os investimentos em malária vivax representaram apenas 3,1% de todo capital utilizado em pesquisa sobre malária no mundo (PATH, 2011).

Tradicionalmente considerada uma infecção benigna, a infecção por *P. vivax* tem sido implicada em casos de malária grave associados principalmente a quadros de anemia grave, trombocitopenia grave, choque circulatório e disfunção hepática (RAHIMI et al., 2014). Os casos de malária vivax grave são classificados pela presença de achados clínicos e laboratoriais que são definidos por critérios de gravidade organizados pela Organização Mundial de Saúde (OMS) para a malária falciparum (WHO, 2010). Não existem critérios específicos para o *P. vivax* (WHO, 2010). Estudos prospectivos na Papua, Indonésia, demonstraram uma taxa de

mortalidade de 1,6% entre pacientes hospitalizados com malária vivax (ANSTEY et al., 2007; TJITRA et al., 2008). Na Venezuela, tem se verificado o aumento de óbitos em crianças menores de dez anos relacionados à infecção por *P. vivax* (RODRIGUEZ-MORALES; BENÍTEZ; ARRIA, 2008). Em recente metanálise com estudos publicados entre 1900 a 2014, a taxa de letalidade estimada entre os pacientes infectados com *P. vivax* foi de 0.3% (RAHIMI et al., 2014). No Brasil, de 2000 a 2013, o Ministério da Saúde informou oficialmente 121.527 internamentos e 1.399 mortes atribuídas à malária (sem especificação da espécie) (MS, 2014). Nos últimos anos têm sido relatadas complicações graves da infecção por *P. vivax* no Brasil, com a descrição de manifestações hematológicas, respiratórias, cerebrais, renais, incluindo óbitos (ALEXANDRE et al., 2010; ANDRADE et al., 2010a; DE LACERDA; DE OLIVEIRA; ALECRIM, 2007; LACERDA; HIPÓLITO; PASSOS, 2008).

A hiperbilirrubinemia (bilirrubina total > 3.0mg/dL), também chamada clinicamente de icterícia, é descrita em alguns estudos como o achado mais comum em crianças e adultos com malária vivax considerada grave (KOCHAR et al., 2009, 2010). Contudo, a definição mais recente de málaria grave da OMS não considera a hiperbilirubinemia isolada como um critério de gravidade, exceto nos casos em que seja acompanhada por outra disfunção de órgãos vitais (WHO, 2010). A fisiopatologia da icterícia não é bem conhecida, mas a hemólise intravascular e o dano hepático associado à malária parecem contribuir para esta manifestação (ANAND; PURI, 2005). Vale ressaltar que outras doenças que evoluem com uma síndrome ictérica e

que são comuns em regiões endêmicas para malária precisam ser excluídas, como a leptospirose, febre tifóide e hepatites (LACERDA et al., 2012).

O espectro clínico da malária é diverso e abrange desde uma infecção sintomática grave ou não-complicada até uma doença assintomática. Estas diferentes manifestações dependem: da virulência do parasita; da susceptibilidade e resposta imune do hospedeiro humano; dos mecanismos de tolerância da doença; e dos fatores ambientais (ANDRADE; BARRAL-NETTO, 2011; MEDZHITOV; SCHNEIDER; SOARES, 2012). Embora não haja nenhuma definição padronizada para malária assintomática, esta pode ser definida pela presença do parasita (parasitemia positiva) sem desenvolver sintomas clínicos da doença durante um certo período (LINDBLADE et al., 2013). A malária assintomática tem sido um problema mundial visto que os indivíduos servem como reservatórios do parasita e mantêm a transmissão da doença através da produção de gametócitos, sendo grandes obstáculos para a erradicação da malária (WHITE, 2008). Em estudo na Região Amazônica Brasileira, indivíduos com malária vivax assintomática dificultam a eliminação da malária residual em comunidades com baixa incidência baixa da doença por representarem a maioria dos casos de malária (56.6%) e mais de 90% deles produzirem gametócitos (BARBOSA et al., 2014).

1.3 FISIOPATOLOGIA DA MALÁRIA

1.3.1 Via do Heme

A hemólise intravascular que ocorre na fase sanguínea da malária está associada à liberação do conteúdo de hemoglobina (Hb) dos eritrócitos. Na presença de espécies reativas de oxigênio (ERO) e nitrogênio presentes na

circulação, os dímeros livres de Hb são oxidados para a forma de metahemoglobina, com liberação do seu grupo prostético, o heme (BALLA et al., 1993, 2000; JENEY et al., 2002). O heme livre possui diversos efeitos danosos ao ser humano (FERREIRA et al., 2008). Esta molécula possui citotoxicidade aumentada para as células endoteliais, expondo a matrix subendotelial pró-trombótica à cascata de coagulação com formação de graus variados de trombos microvasculares e concomitante vaso-oclusão e isquemia tecidual (JENEY et al., 2002). O heme pode também atuar como quimioatraente celular dos polimorfonucleares, com participação na ativação e sobrevivência celular, estimulando, consequentemente, a geração de ERO maléficas ao ser humano (ARRUDA et al., 2004; GRAÇA-SOUZA et al., 2002; PORTO et al., 2007). Outros efeitos danosos do heme foram descritos no seu papel em sensibilizar células não-hematopoiéticas a submeter-se a apoptose mediada por fator de necrose tumoral (TNF) (SEIXAS et al., 2009) e suprimir mediadores anti-inflamatórios, como a prostaglandina E2 (PGE2) e o fator de transformação do crescimento-β (TGF-β) (ANDRADE et al., 2010d).

O plasmódio desenvolveu uma série de mecanismos para proteção contra os efeitos deletérios do heme livre, com destaque para a polimerização do heme em hemozoína, um pigmento malárico que neutraliza os efeitos pró-oxidativos do átomo de ferro (Fe) presente na molécula (FRANCIS; SULLIVAN; GOLDBERG, 1997). A relevância deste mecanismo de defesa é corroborada pelos efeitos terapêuticos de drogas anti-maláricas, como por exemplo a cloroquina, que inibem a formação de hemozoína (SLATER; CERAMI, 1992). Com base nessa estratégia, o parasita consegue se replicar eficientemente dentro dos eritrócitos, que posteriormente serão

hemolisados com liberação de Hb na circulação (FRANCIS; SULLIVAN; GOLDBERG, 1997).

O hospedeiro também criou artifícios para se proteger da citotoxicidade da Hb e heme livres. Sob condições homeostáticas, a Hb pode rapidamente se ligar à haptoglobina (Hp), proteína plasmática tetramérica (α2β2) (FERREIRA et al., 2008). A Hp é considerada uma proteína de fase aguda que aumenta 2 a 4 vezes em resposta a uma inflamação aguda (IMRIE et al., 2007). O heme livre também pode se ligar à hemopexina ou albumina, porém com menor afinidade. O complexo Hb-Hp é reconhecido e internalizado via receptor grupamento de diferenciação (CD)163 expresso em monócitos/macrófagos na polpa vermelha do baço (KRISTIANSEN et al., 2001). O CD163, por sua vez, pode ser liberado da membrana na forma de uma glicoproteína solúvel plasmática (sCD163) possivelmente em resposta a estímulos inflamatórios e estresse oxidativo (TIMMERMANN; HÖGGER, 2005). O sCD163 parece inibir a ativação de linfócitos T humanos induzida por forbol éster, resultando na atenuação da resposta imune (HÖGGER; SORG, 2001). Os níveis de sCD163 são mais elevados em indivíduos com malária não complicada quando comparados àqueles com a forma grave, assim como nos indivíduos infectados com malária de qualquer forma clínica quando comparados a indivíduos que compõem o grupo controle saudável (KUSI et al., 2008).

Após a internalização do complexo Hb-Hp, o heme será degradado via a enzima intracelular heme oxigenase-1 (HO-1). A cadeia beta (β) da Hp possui aproximadamente 40 kDa, e a cadeia alfa (α) é sintetizada por dois alelos variantes: o Hp1 (α1), com aproximadamente 8,86 kDa, que são

subdivididos em 1S e 1F, e o Hp2 (α2), com aproximadamente 17,3 kDa (MAEDA et al., 1984). Esses alelos apresentam afinidades diferentes pela Hb livre (Hp1.1>Hp1.2>Hp2.2), assim como pelo CD163 (Hp2.2>Hp1.2>Hp1.1) (KRISTIANSEN et al., 2001). Além disso, a presença do genótipo Hp2.2 tem sido associado a um aumento do estresse oxidativo quando comparado a presença do genótipo Hp1.1 (ASLEH et al., 2005; MELAMED-FRANK et al., 2001). O genótipo Hp2.2 têm sido associado à susceptibilidade a diversas condições inflamatórias, incluindo malária (ATKINSON et al., 2006; CHEN et al., 2011b; COX et al., 2007; FRIIS et al., 2003; KASVOSVE et al., 2000). Contudo, o genótipo Hp2.2 também foi associado a uma menor propensão de crianças desenvolverem formas graves da malária no Quênia (ATKINSON et al., 2007).

A HO-1 é codificada pelo gene *HMOX1* (heme oxigenase [decíclica] 1) e é induzida ubiquamente sob estresse oxidativo (SEIXAS et al., 2009). Esta enzima cinde o heme liberando a protoporfirina IX e o átomo de Fe, com geração de monóxido de carbono (CO) (FERREIRA et al., 2008). A protoporfirina IX é transformada em biliverdina, que pode ser convertida pela biliverdina redutase em bilirrubina (STOCKER et al., 1987). A infecção pelo plasmódio em humanos está associada à indução de níveis elevados de HO-1 (MEDANA et al., 2001; SCHLUESENER; KREMSNER; MEYERMANN, 2001). Entretanto, em modelos experimentais de malária, a indução de HO-1 está associada a uma tolerância maior ao plasmódio como resultado da capacidade da HO-1 em controlar o dano tecidual não-específico e a imunopatologia através da redução da inflamação (PAMPLONA et al., 2007). Essa proteção ocorre através da produção de CO, que se liga com afinidade

alta à Hb livre, prevenindo a liberação de heme na circulação (PAMPLONA et al., 2007). De modo similar, observou-se que a ocorrência de uma deleção no gene da HO-1 (*HMOX1*) está associada ao desenvolvimento de malária grave em camundongos (SEIXAS et al., 2009). Adicionalmente, o mecanismo de proteção de individuos com anemia falciforme contra a infecção malárica parece ser através da indução da HO-1 em modelo experimental (FERREIRA et al., 2011).

Um polimorfismo microsatellite (GT)n na região promotora do gene *HMOX1* está associada à síntese aumentada ou diminuída da HO-1 em resposta a diferentes estímulos. Os indivíduos com a repetição menor do dinucleotídeo (GT)n apresentam expressão maior da HO-1, enquanto que a repetição maior está associada à síntese diminuída da enzima (EXNER et al., 2004). Este polimorfismo já foi descrito em diversas doenças como: doença de Parkinson e Alzheimer (KIMPARA et al., 1997), doença coronariana arterial (CHEN et al., 2002), enfisema (YAMADA et al., 2000), câncer (HU et al., 2010), insuficiência respiratória (SHEU et al., 2009), nefropatia (CHIN et al., 2009) e artrite reumatóide (WAGENER et al., 2008). Na malária, estudos demonstraram associações entre repetições (GT)n curtas e a malária falciparum grave (TAKEDA et al., 2005; WALTHER et al., 2012), enquanto que outro estudo não encontrou associação entre o polimorfismo microsatellite da *HMOX1* e susceptibilidade à malária (KUESAP et al., 2010).

1.3.2. Hepcidina

O heme é degradado pela HO-1 produzindo biliverdina, CO e ferro lábil (Fe2+). As concentrações de ferro são controladas através do mecanismo de

feedback da hepcidina, hormônio que regula a homeostase do ferro pela degradação do exportador de ferro, a ferroportina, prevenindo a liberação do ferro reciclado pelos eritrócitos e do ferro da dieta pelos macrófagos e enterócitos, respectivamente (GANZ, 2006). Mutações no gene da *ferroportina* (*SLC40A1* [família de carreador solúvel 40 membro 1]) com disfunção do exportador têm sido associadas com tendência a acúmulo de ferro e hemocromatose em humanos (KASVOSVE et al., 2005; MONTOSI et al., 2001; WALLACE et al., 2002). O acúmulo de Fe intracelularmente é perigoso visto que causa estresse oxidativo, a menos que se ligue à ferritina, que atua como uma molécula antioxidante em diversos modelos (BALLA et al., 1992; BERBERAT et al., 2003; COZZI et al., 2000). Os níveis de ferritina estão diminuídos em indivíduos com *P. vivax* da Amazônia Brasileira de modo diretamente proporcional ao dano hepático (GOZZELINO et al., 2012). Adicionalmente, em modelo experimental, a ferritina promove tolerância à malária por prevenir que o ferro lábil intracelular induza a ativação da molécula pró-apoptótica quinase c-Jun N-terminal (JNK), e esta ação requer a expressão de HO-1 (GOZZELINO et al., 2012).

Através da hepcidina, o ferro do metabolismo do heme nos macrófagos e monócitos é retido intracelularmente; no entanto, nos casos de excesso do hormônio e saturação da ferritina, pode acontecer o acúmulo e impregnação pelo ferro de alguns órgãos, especialmente o fígado, baço e coração (DE MAST et al., 2009a). Estudos têm demonstrado que os níveis da hepcidina estão aumentados na malária humana sintomática ou assintomática tanto pelo *P. falciparum* quanto pelo *P. vivax* (AYOYA et al., 2009; DE MAST et al., 2009a, 2009b, 2010; HOWARD et al., 2007). Além

disso, a hepcidina demonstrou ser o melhor preditor de absorção de ferro pelos eritrócitos em crianças com comorbidades (anemia, deficiência de ferro e infecções), sugerindo o potencial uso desse hormônio como biomarcador em programas de suplementação de ferro (PRENTICE et al., 2012).

A síntese da hepcidina é induzida pelo ferro da dieta, estoques de ferro e inflamação, e é suprimida pela deficiência de ferro, hipóxia e eritropoiese (GANZ, 2011). O mediador inflamatório mais bem descrito em induzir a expressão da hepcidina é a interleucina (IL) 6 (IL-6) (NEMETH et al., 2004). Em modelo experimental de malária, a produção de hepcidina previne uma infecção subsequente provavelmente por reduzir os níveis de ferro necessários para o completo desenvolvimento do plasmódio, e este efeito é inibido em camundongos tratados com anticorpos anti-IL-6 (PORTUGAL et al., 2011). O IL-10 também parece induzir a hepcidina em macrófagos co-culturados com eritrócitos infectados pelo *P. falciparum* através da fosforilação do tradutor de sinal e ativador de transcrição (STAT)-3 (HUANG et al., 2014). De modo interessante, hemácias infectadas pelo plasmódio induzem a síntese de ácido ribonucléico mensageiro (RNAm) de hepcidina em células mononucleares de sangue periférico, sugerindo um papel de células circulatórias do sistema imune na produção deste hormônio (ARMITAGE et al., 2009).

1.3.3. Resposta Imune

A resposta imune do indíviduo tem um papel importante na patogênese das manifestações clínicas da malária assim como na tolerância ou resistência ao parasita. Indivíduos da área endêmica para malária podem

adquirir imunidade parcial ao plasmódio: imunidade anti-doença pode prevenir o desenvolvimentos de sintomas clínicos da patologia apesar da presença do plasmódio; e imunidade anti-parasita, que suprime a carga parasitária (DAUBERSIES et al., 1996; DAY; MARSH, 1991; TRAPE et al., 1994). Normalmente, os indivíduos com malária assintomática são mais velhos e apresentam número maior de episódios prévios de malária, sugerindo uma imunidade protetora ao longo dos anos de exposição ao plasmódio na área endêmica (ANDRADE et al., 2009). A malária grave, por sua vez, está mais associada com crianças e indivíduos primoinfectados, sugerindo uma resposta imune ineficaz contra a infecção (DAY; MARSH, 1991).

A resposta imune é montada quando os patógenos são reconhecidos por células do sistema imune inato (células dendríticas, macrofagos, neutrófilos, células *natural killer*, basófilos, eosinófilos e mastócitos) através dos receptores de padrões moleculares associados á patógenos (PAMPs), sendo o receptor tipo toll (TLRs) o mais importante. Através dessa interação inicial entre o hospedeiro e o patógeno, o sistema imune inato organiza uma defesa imediata contra o organismo invasor e também ajuda na direção de uma resposta de células do sistema imune adaptativo (células B e T), que reconhecem e ligam antígenos estranhos através de um repertório de receptores de superfície. As interações receptor-ligante direcionam células do sistema imune adaptativo e inato a transcrever seus genes na forma de RNAm; o RNAm é, em seguida, traduzido em proteínas intracelulares, proteínas de membrana e proteínas secretórias, que juntas formam a qualidade da resposta imune contra o micróbio invasor (TRAN et al., 2012).

1.3.3.1. *Imunidade inata*

As células da resposta imune inata são a primeira linha de defesa do organismo contra patógenos. A atividade funcional de neutrófilos e macrófagos/monócitos já foi descrita em mediar a resposta imune de indivíduos com malária por *P. falciparum* (CHIMMA et al., 2009; JOOS et al., 2010). Especificamente na malária vivax aguda, monócitos e neutrófilos estão altamente ativados; enquanto que os monócitos são a maior fonte de citocinas em resposta a agonistas de TLR, neutrófilos demonstram ter uma maior atividade fagocítica e produção de superóxido (LEORATTI et al., 2012). Dentre os diferentes subtipos de monócitos classificados com base na expressão de CD14 e CD16 (CD14+CD16-, clássico; CD14+CD16, inflamatório; CD14dimCD16+, patrulhador), o subtipo inflamatório está associado com a produção elevada de citocinas pró-inflamatórias, expressão de receptores de quimiocinas e moléculas de adesão, assim como é o mais eficiente em fagocitar reticulócitos infectados e produzir ERO durante a malária por *P. vivax* (ANTONELLI et al., 2014).

1.3.3.2. *Imunidade adquirida*

As células do sistema imune inato ajudam a direcionar as respostas das células da resposta imune adaptativa. As porcentagens de células T CD4+ e receptor de células T (TCR)γδ estão aumentadas no sangue periférico de indíviduos com malária vivax aguda comparado com grupo controle endêmico (JANGPATARAPONGSA et al., 2012; SILVA et al., 2013). Em estudo na Uganda, células T CD4+ de indivíduos com menos episódios prévios de malária são mais inflamatórias (maior produção de TNF),

enquanto que o perfil de resposta das células T CD4+ daqueles com mais episódios prévios de malária é mais regulatório (com produção de IL-10), sugerindo que a produção de IL-10 pelos células T Th1 podem ajudar a prevenir a imunopatologia da doença através da diminuição da resposta pró-inflamatória (JAGANNATHAN et al., 2014). Igualmente importante, as células T regulatórias (Treg) (CD4+CD25+FOXP3+) parecem mediar seus efeitos através de contato celular direto ou pela indução de citocinas regulatórias, IL-10 ou TGF-β (POWRIE et al., 2003; THORNTON; SHEVACH, 2000). Células Treg são induzidas em resposta à infecção pelo *P. falciparum* ou *P. vivax* e estão associadas a um aumento na produção de TGF-β e diminuição da produção de citocinas pró-inflamatórias, podendo resultar em uma resposta imune mais amena (GONÇALVES et al., 2010; WALTHER et al., 2005).

A resposta imune humoral também é importante no contexto da malária visto que a transferência passiva de imunoglobulina (Ig)G de adultos imunes para crianças e adultos não-imunes com malária aguda rapidamente reduziu a parasitemia e resolveu a febre (COHEN; MCGREGOR; CARRINGTON, 1961; SABCHAREON et al., 1991). De maneira similar à infecção pelo *P. falciparum*, indivíduos com *P. vivax* tem uma percentagem menor de células B (CD19+) quando comparados com o grupo controle endêmico (KASSA et al., 2006). Além disso, nem todas as exposições à malária resultam na geração de células B de memória e anticorpos IgG contra o parasita são de curta duração e falham em aumentar durante uma reinfecção, sendo a memória imunológica um desafio em muitos ensaios de vacinas (BEJON et al., 2006; DORFMAN et al., 2005). Indivíduos com malária assintomática tendem a ter níveis mais elevados de IgG específicos contra

antígenos do plasmódio do que os outros desfechos clínicos da malária, sugerindo contato prévio maior com o parasita (ABDEL-LATIF et al., 2003; BRAGA et al., 2002). De modo semelhante, indivíduos com malária assintomática por *P. vivax* têm concentração maior de anticorpo IgG contra a saliva do *Anopheles darlingi* na região Amazônica Brasileira como resultado da exposição maior ao vetor do plasmódio (ANDRADE et al., 2009).

1.3.3.3. *Mediadores imunes e alterações genéticas*

O balanço entre citocinas e quimiocinas, regulatórias e pró-inflamatórias, assim como alterações genéticas em genes relacionados com a resposta imune parecem ter papéis importantes nos desfechos clínicos da malária. Na infecção por *P. vivax*, níveis elevados das citocinas pró-inflamatórias (interferon [IFN]-γ e TNF) estão aumentados e a concentração da citocina regulatória (IL-10) está diminuida gradualmente com a gravidade da infeccção (malária assintomática, sintomática moderada, e grave); e após o tratamento, ocorre redução significativa de IFN-γ e TNF (ANDRADE et al., 2010a). A patogênese de doenças infecciosas, como a malária, é parcialmente condicionada pela variabilidade na região codificadora e/ou promotora de genes associados à expressão de moléculas inflamatórias (BURGNER et al., 2003; CRAMER et al., 2004; OUMA et al., 2008a, 2008b). Desta forma, o melhor entendimento do processo de patogênese pode ser adquirido através da identificação de polimorfimos funcionais em genes críticos para o desenvolvimento do curso clínico da doença.

O aumento nas concentrações do TNF está relacionado a sintomas associados à patogênese da malária, como a febre, bem como a formas

graves da infecção (KARUNAWEERA et al., 1992; KWIATKOWSKI et al., 1990). Entretanto, o TNF também tem sido associado à presença de uma potente atividade anti-parasitária, assim como níveis elevados persistentes dessa citocina levam à uma rápida melhora da febre e diminuição da parasitemia (DEPINAY et al., 2011; MORDMÜLLER et al., 1997). Em estudo de biomarcadores de gravidade, observou-se que a superóxido dismutase-1 (SOD-1), enzima envolvida no estresse oxidativo, é um marcador com melhor perfil para identificar os casos de malária vivax grave do que os níveis plasmáticos de TNF (ANDRADE et al., 2010b). Polimorfismos no gene do *TNF* podem estar associados a uma expressão diferenciada desta citocina e a susceptibilidade ou resistência maior a malária. Alterações genéticas no *TNF* são descritas em diferentes populações no mundo; entretanto, com alguns resultados contraditórios. O polimorfismo de nucleotídeo único (SNP) *TNF*-308G>A, por exemplo, tem sido associado a maior susceptibilidade, resistência ou sem associações com malária grave por *P. falciparum* (CLARK et al., 2009; MCGUIRE et al., 1994; MEYER et al., 2002; WATTAVIDANAGE et al., 1999). Em estudo com malária vivax na Região Amazônica Brasileira, um haplótipo do *TNF* (*TNF*-1031T/-863A/-857T/- 308G/-238G), que inclui o alelo G do SNP *TNF*-308, está associado com maior susceptibilidade à doença (SORTICA et al., 2012).

Os níveis aumentados de IL-6 estão associados à malária grave e à incidência aumentada de episódios clínicos da doença (DAY et al., 1999; ROBINSON et al., 2009; WENISCH et al., 1999). A β-hematina, análogo sintético da hemozoína, e os eritrócitos infectados estimulam a liberação de IL-6, assim como a de IL-10 e TNF-α (GIUSTI et al., 2011). Dentre os

polimorfismos presentes na região promotora do gene da IL-6, existe o *IL6*-176 G>C em que o alelo C está associado ao aumento na expressão de IL-6 em neonatos e adultos com reações inflamatórias agudas (BRULL et al., 2001; KILPINEN et al., 2001). A frequência dos genótipos CG/GG do SNP *IL6*-176 G>C é maior nos grupos étnicos não-Fulani, que apresentam maior susceptibilidade à malária, nos casos de malária sintomática ou assintomática por *P. falciparum* (ISRAELSSON et al., 2011).

A proteína nuclear complexo principal de histocompatibilidade (HLA)-B-associada ao transcrito 1 (BAT1) é uma helicase de RNA codificada pelo gene *DDX39B* (caixa polipeptídica 39B DEAD [Asp-Glu-Ala-Asp]) e modula a expressão de citocinas pró-inflamatórias, como a TNF e o IL-6 (ALLCOCK; WILLIAMS; PRICE, 2001). Os SNPs na região promotora da *DDX39B* (-22C>G e -348C>T) parecem modificar a atividade transcricional e a ligação de fatores nucleares de transcrição, como os oligonucleotídeos YY1 e Oct1 (PRICE et al., 2004). As sequências do ácido desoxirribonucléico (DNA) com os alelos -22G e -348T do *DDX39B* são mais expressas do que com os alelos -22C e -348C (PRICE et al., 2004). Além disso, o *DDX39B* está localizado no mesmo cromossomo que o *TNF* e polimorfismos nestes genes podem ter efeitos complementares ou aditivos na modulação da resposta imune (FLORI et al., 2005). Mutações no gene *BAT1* foram descritas em diversas doenças com perfil inflamatório, incluindo neuropatia (CHERRY et al., 2008), miastenia gravis (DEGLI-ESPOSTI; LEELAYUWAT; DAWKINS, 1992), doença de Alzheimer (GNJEC et al., 2008), infarto do miocárdio (KOCH et al., 2007), hepatite autoimune (OLIVEIRA et al., 2011), artrite reumatóide (QUIÑONES-LOMBRAÑA et al., 2008), e doença de Chagas

(RAMASAWMY et al., 2006). Contudo, os polimorfismos no *DDX39B* (-22C>G e -348C>T) e o papel da BAT1 ainda não foram estudados no contexto da malária.

1.3.4. Papel das Coinfecções

Coinfecções podem modular ou intensificar a resposta imune de indivíduos com malária. É descrito que indivíduos com malária assintomática normalmente possui múltiplos clones do *Plasmodium sp.* geneticamente distintos, e a infeção multiclonal pode ser um marcador de imunidade e conferir proteção contra a doença por induzir uma resposta imune mais abrangente (RONO et al., 2013). Em relação aos outros patógenos, a coinfecção com hepatite B está associada com a malária vivax assintomática e pontecializa a resposta imune protetora desses indivíduos através da produção de níveis baixos de TNF e da relação inflamatória IFN-γ/IL-10 e concentração alta de IL-10 (ANDRADE et al., 2011). Além disso, uma infecção pré-existente pela filária (*Wuchereria bancrofti* ou *Mansonella perstans*) atenua a resposta imune associada com a malária grave por *P. falciparum* e protege contra anemia (DOLO et al., 2012).

No que se refere aos helmintos, coinfecções com *Ascaris lumbricoides* ou *Schistosoma haematobium* exibem uma tendência de proteção, enquanto que coinfeções com ancilóstomo ou *Schistosoma mansoni* levam a piora clínica da doença e maior incidência de malária (ADEGNIKA; KREMSNER, 2012). Em um estudo prospectivo, a coinfeção do *P. falciparum* com o *Schistosoma haematobium* foi significantemente associada com o risco reduzido de malária aguda no seguimento de indivíduos com malária

assintomática (DOUMBO et al., 2014). As infecções por vírus, por sua vez, parecem exibir uma tendência de aumentar a gravidade quando associadas com a malária. A coinfecção entre o virus da imunodeficiência humana (HIV) e o *P. falciparum* aumenta a gravidade e mortalidade por malária (BERG et al., 2014). Adicionalmente, indivíduos infectados com o HIV e portadores de malária assintomática podem ter aumento da carga viral, o que pode acelerar a progressão e gravidade da doença (VERHOEFF et al., 1999; WHITWORTH et al., 2000). A coinfecção do *P. falciparum* com o parvovírus humano B19 também contribui para uma malária mais complicada, sobretudo por anemia grave (TOAN et al., 2013). Em estudo sobre infecção concomitante do *P. vivax* e dengue na Amazônia Brasileira, pacientes coinfectados tiveram chances maiores de desenvolverem doença grave, sangramento profundo, hepatomegalia e icterícia comparados com as monoinfecções (MAGALHÃES et al., 2014).

1.4 AVANÇOS NAS ANÁLISES DE BIOMARCADORES EM MALÁRIA

Um biomarcador pode ser definido como qualquer parâmetro que pode ser usado como um indicador de uma doença em específico ou outro processo fisiológico (ANDRADE; BARRAL-NETTO, 2011). Os biomarcadores podem ser convencionalmente classicados de acordo com o propósito de diagnóstico ou de gravidade, e sinais/sintomas clínicos, marcadores laboratórios (proteínas, metabólitos, polimorfismos genéticos) são alguns exemplos (ANDRADE; BARRAL-NETTO, 2011). A grande parte dos estudos realizados sobre a resposta imune na malária por *P. vivax* avaliam uma ou um pequeno conjunto de biomarcadores relacionados com o sistema imune, sem uma avaliação das suas inter-relações. Como as ações das células

imunes, mediadores inflamatórios e alterações genéticas são interdependentes, os estudos que singularizam moléculas perdem uma parte importante do entendimento do funcionamento da resposta imune.

Grandes quantidades de dados estão ficando disponíveis com os recentes avanços tecnológicos em métodos de pesquisa. Antigamente, poucas proteínas eram mensuradas por ELISAs (ensaio imunoadsorvente ligado à enzima) específicos, e, atualmente, as técnicas de citometria de fluxo e multiplex permitem a dosagem de dezenas de diferentes mediadores em um único ensaio. Além disso, recentemente, foi possível identificar diversas proteínas e metabólitos por proteômica e metabolômica, e desvendar o código genético através das técnicas de sequenciamento de DNA e RNA, e microarranjos (TRAN et al., 2012). Com a idéia da biologia de sistemas e dados de métodos de alto rendimento, a identificação de biomarcadores requer uma análise sofisticada de maneira integrada e conectada (TRAN et al., 2012). Contudo, um grande desafio na descoberta de biomarcadores confiáveis é escolher o melhor tipo de análise, incluindo ferramentas de bioinformática, para extrair a melhor informação dos dados.

Quando se trabalha com múltiplas variáveis, normalmente é necessário utilizar métodos de análise multivariada: análise do componente principal (ACP), agrupamento hierárquico e análises discriminatórias são alguns exemplos. ACP, assim como o escalonamento multidimensional, são métodos que têm sido usados para reduzir a dimensionalidade de dados multivariados, identificando agrupamentos e detectanto variáveis que podem explicar a estrutura de variação dos dados. Além disso, o agrupamento hierárquico e os *heat maps* também têm sido usados no contexto da malária

para melhor visualizar grande volume de dados em uma plotagem bidimensional e identificar agrupamentos associados ao desfecho clínico da doença, a resposta imunológica e a biologia do vetor (PORTUGAL et al., 2014; SÁNCHEZ-ARCILA et al., 2014). Em estudo de proteômica com o uso de espectometria de massa, a análise da expressão de proteínas do *P. falciparum* por agrupamento hierárquico e ACP permitiu classificar as amostras em dois grupos que correspondiam aos grupos do estudo (malária na gravidez e casos de malária aguda); e o primeiro componente principal contou com 41% da variação total, mostrando uma separação clara entre os grupos (BERTIN et al., 2013). Igualmente importante, em estudo sobre coinfecção com malária e HIV, um painel vasto de dosagens plasmáticas de citocinas, quimiocinas e fatores de crescimento foi reduzido em menores grupos por ACP, e os diferentes componentes principais que surgiram demonstraram ser diferentes de acordo com a positividade para o HIV (DAVENPORT et al., 2012). Biomarcadores baseados em sintomas também podem ser analisados de maneira integrada: o primeiro componente obtido através da ACP demonstrou maior valor para um grupo de sintomas, que explicaram 32.1% da variabilidade sintomatólogica nos pacientes; e este componente foi usado como um index clínico de malária grave (SORTICA et al., 2014). Neste mesmo contexto de métodos multivariados, a análise da função discriminatória, em que uma variável de agrupamento, que pode ser encontrada em diferentes grupos de malária, pode ser prevista por uma ou mais variáveis (biomarcadores). Por meio da análise discriminatória, identificou-se que a ornitina urinária tem o potencial de ser utilizado como biomarcador de malária por *P. vivax* quando comparados com indivíduos com

outras síndromes febris (SENGUPTA et al., 2011). Portanto, um dos objetivos dos métodos multivariados em estudos de biomarcadores é o de tentar encontrar bioassinaturas moleculares e identificar variáveis importantes, em um painel vasto de moléculas, que possam explicar o agrupamento dos dados.

A abordagem por inteligência artificial tem sido empregada para analisar grupos de biomarcadores através das redes neurais (RN) artificiais. Estas redes estão sendo aplicadas, nos últimos anos, para fazer tarefas complexas em diferentes áreas, como em estratégias dos sistemas de modelagens, que podem ser concebidas como um grupo de entradas e saídas de dados (CUNHA et al., 2010). A estrutura de uma RN pode ser visualizada como uma simplificação das propriedades complexas dos neurônios biológicos, em que as ligações e conexões entre os neurônios são modelados simulando os axônios e dendritos encontrados em redes de neurônios biológicos (BACHTIAR et al., 2011). A mudança no potencial da membrana celular representa a transferência de dados através da via de informação biológica e permite extrair as características essenciais do conjunto de dados e fazer uso dessa informação para predizer valores (BACHTIAR et al., 2011). As RN já foram aplicadas na malária: predição de casos de malária vivax por dados ambientais (KIANG et al., 2006; SHI et al., 2014); estrutura do biosensor olfatório de mosquitos *Anopheles gambiae* (BACHTIAR; UNSWORTH; NEWCOMB, 2013); e identificação de epítopos de malária para células T (BRUSIC et al., 2001). Na infecção por *P. vivax*, o uso das RN permitiu criar um método alternativo mais acurado de diagnóstico da malária (ANDRADE et al., 2010c).

A análise em rede pode ser utilizada como mais uma maneira de se identificar biomarcadores confiáveis de gravidade ou resistência. Uma estrutura inferencial por rede é gerada a partir dos valores de cada biomarcador em um programa de análise de dados, sendo que este programa seleciona os padrões de distribuição dos mediadores associados com cada grupo clínico e identifica interações entre as moléculas. Cada biomarcador é selecionado como um alvo e o programa procura entre os outros marcadores quais estão associados com o alvo, usando para isto o teste de correlação de Spearman. Como resultado, cada biomarcador é representado com um ícone e as moléculas relacionadas com o alvo selecionado são ligados por um traço. A abordagem por redes pode ser usada para visualizar a interação entre SNPs; um estudo associou polimorfismos em genes relacionados com a resposta imune e a malária na placenta por análise em rede (SIKORA et al., 2011). As análises em redes Bayesianas (RB) também é usada para modelar os efeitos dos biomarcadores e suas interações em diferentes desfechos. As RB são modelos probabilísticos que descrevem dependências condicionais estatísticas entre múltiplas variáveis (PINEDA-PEÑA et al., 2014) e essas dependências são representadas em uma figura acíclica, que representa o componente qualitativo das RB (DEFORCHE et al., 2006). Em estudo sobre resistência transmitida por medicamentos em população positiva para o HIV, identificou-se por RB que o subtipo B do vírus é o marcador com melhor predição para esta resistência (PINEDA-PEÑA et al., 2014). Deste modo, o uso das redes no contexto dos biomarcadores pode ser uma ferramenta

adicional para melhor visualizar as correlações e entender o padrão de interações entre as moléculas.

2. JUSTIFICATIVA

A malária é um problema de saúde pública no Brasil com milhares de pessoas anualmente infectadas, em sua maioria, pelo *P. vivax*. Diversas são as manifestações clínicas associadas à malária, incluindo malária grave, hiperbilirrubinemia isolada, malária não-complicada e malária assintomática. Neste espectro, a identificação de biomarcadores confiáveis tanto de gravidade ou resistência são indispensáveis para o auxílio no seguimento, diagnóstico e terapêutica da malária. Esta situação é ainda mais necessária na malária vivax, em que não existe um critério padronizado de sinais/sintomas de gravidade; e os portadores assintomáticos, frequentemente encontrados na Região Amazônica Brasileira, são os maiores obstáculos para a eliminação local da doença.

A resposta imune dos indivíduos com *P. vivax* permanece pouco conhecida, e as vacinas até então formuladas não têm memória imunológica ou não são antigênicas. A melhor caracterização do sistema imune na malária é pré-requisito para os esforços de controle e eliminação da doença. Além disso, esta doença exerce pressão seletiva significativa no genoma humano, sendo a força evolucionária por trás da doença falciforme, talassemias e deficiência de glicose-6-fostato. A investigação de variações genéticas permite identificar as vias moleculares críticas na patogênese e imunidade, possibilitando a geração de estratégias novas voltadas para o tratamento e/ou prevenção de doenças.

Esta Tese é composta por 5 artigos originais e 2 artigos de revisão que permitem elucidar melhor a imunopatogênese da malária pelo *P. vivax* com enfoque na identificação de biomarcadores imunológicos e genéticos associados aos diferentes desfechos clínicos da doença. Nós identificamos alterações genéticas que podem diminuir a defesa humana e aumentar os efeitos nocivos do heme livre, sugerindo que para a identificação de novos alvos terapêuticos nessa via é necessário entender o componente genético humano. Compreender a resposta imune de indivíduos com malária vivax é um dos pilares para o desenvolvimento de vacinas eficazes; e nosso grupo de pesquisa descobriu assinaturas imunológicas associadas com a infecção pelo *P. vivax* por uma análise em redes, assim como encontrou polimorfismos que podem influenciar esta resposta imune.

Em revisão recente dos critérios definidores de malária falciparum grave, a OMS retirou a hiperbilirrubinemia isolada como fator de gravidade. Contudo, pouco se sabe sobre a resposta imune desses indivíduos, e nós verificamos que esta é parecida com aquela de indivíduos com malária não-complicada, sugerindo uma doença menos grave. Desta maneira, esses dados podem ajudar profissionais de saúde no manejo de pacientes infectados pelo plasmódio e com hiperbilirrubinemia isolada. Ainda no contexto clínico, sabe-se que diferentes doenças infecciosas podem ter sintomas semelhantes, caracterizados por uma síndrome febril aguda. Este é o caso da malária e da dengue, que podem ser confundidas na prática clínica ou em alguns casos podem, inclusive, estar associadas. Nós propomos um algoritmo clínico baseado em dados laboratoriais simples (hemoglobina, plaquetas, enzimas hepáticas) que pode auxiliar a equipe de saúde no

diagnóstico diferencial dos casos de monoinfecções ou coinfecção malária/dengue em regiões endêmicas.

Em conjunto, os manuscritos desta Tese ajudam no entendimento dos mecanismos imunopatológicos na infecção pelo *P. vivax* e podem guiar e auxiliar futuros estudos sobre métodos diagnósticos, terapêuticas e vacinas na malária vivax.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Identificar os fatores de gravidade e resistência na malária causada pelo *P. vivax* por meio da avaliação de biomarcadores plasmáticos e genéticos associados com a via do heme, hepcidina e resposta imune em indivíduos com diferentes manifestações clínicas (sintomática, assintomática, sintomática com hiperbilirrubinemia, e grave), assim como nos casos de coinfecção com dengue, da Região Amazônica Brasileira.

3.2 OBJETIVOS ESPECÍFICOS

- Associar níveis plasmáticos e polimorfismos em moléculas associadas à via de metabolização do heme com manifestações clínicas distintas de indivíduos infectados pelo *P. vivax*;

- Comparar o padrão e complexidade da resposta imune e inflamatória, mensurada por biomarcadores sorológicos, em diferentes grupos de pacientes com malária vivax através de uma abordagem analítica por redes;

- Associar a frequência de SNPs em genes relacionados à resposta imune com dosagens de marcadores inflamatórios e desfechos clínicos da malária vivax;

- Descrever as associações entre os níveis da hepcidina e mediadores imunes em indivíduos com malária moderada, malária com hiperbilirrubinemia isolada e malária grave, por *P. vivax*;

- Descrever o padrão da resposta imune (citocinas, quimiocinas) e laboratorial em indivíduos coinfectados com o vírus da dengue e o *P. vivax*.

4. METODOLOGIA

Resumo geral da metodologia empregada para a amostragem dos indivíduos estudados nos manuscritos II, III e IV

Os indivíduos das comunidades de Demarcação e Buritis do estado de Rondônia, na Região Amazônica Brasileira, foram recrutados entre 2006 e 2007. Detecção ativa e passiva da malária foi realizada: visita aos domicílios em áreas de transmissão alta, e pessoas que procuraram atendimento médico nos centros de diagnóstico da Fundação Nacional de Saúde (FUNASA) ou no Hospital Municipal de Buritis. Indivíduos de ambos os sexos, com idade entre cinco a 70 anos, que moraram na área endêmica por mais de seis meses, foram convidados a participar. Os casos de malária foram diagnosticados localmente por microscopia e confirmado por reação em cadeia da polimerase (PCR) *nested* na FIOCRUZ, Salvador, Brasil. Os critérios de exclusão foram: infecção pelo *P. falciparum*, hepatites virais (hepatites A, B, C, D) documentadas, alcoolismo crônico, infecção pelo HIV, febre amarela, leptospirose, câncer ou doenças degenerativas crônicas, traço falciforme e o uso de drogas hepatotóxicas ou imunosupressoras.

Os indivíduos sem sintomas clínicos de malaria foram ativamente recrutados em suas residências por procura ativa, a maioria em comunidades ribeirinhas, e tiveram amostras colhidas para o exame da gota espessa e uma pequena alíquota de sangue em tubos com EDTA (ácido etilenodiamino tetra-acético) para diagnóstico por *nested* PCR. Os diagnósticos desses participantes foram realizados após uma visita clínica e se o teste fosse positivo para malária, uma segunda visita após 30 dias seria realizada para

verificar o surgimento de sintomas sugestivos de malária. Uma nova coleta de amostras foi feita e uma segunda rodada de testes diagnósticos foi realizada. Aqueles que permaneceram com *nested* PCR positivo para *Plasmodium vivax* durante os 30 dias e não apresentaram sintomas clínicos relacionados a malária, como febre (temperatura axilar >37.8°C), calafrios, sudorese, artralgia, cefaléia intensa, náuseas, vómitos, icterícia e astenia grave durante este período foram considerados como casos assintomáticos de malária. Durante a primeira visita clínica (previamente aos resultados dos testes diagnósticos), todos os indivíduos assintomáticos receberam aconselhamento e foram orientados a procurar serviço de saúde em um centro de referência para malária se os sintomas aparecessem durante o período entre as duas visitas. Após a segunda visita clínica, todos os pacientes que tinham o teste positivo para malária por microscopia e/ou *nested* PCR foram tratados conforme o guia de tratamento do Ministério da Saúde brasileiro (MS, 2014). As amostras de sangue para estudar os biomarcadores foram coletadas durante a segunda visita clínica, antes do início dos medicamentos anti-maláricos. Indivíduos sintomáticos foram prontamente tratados. Um total de 530 indíviduos foi estudado, estratificado nos seguintes grupos: não-infectados (n=176), malária vivax assintomática (n=148), malária vivax sintomática (n=187), malária grave sem mortalidade (n=13), e malária grave que evoluíram para o óbito (n=6). As amostras de sangue obtidas dos pacientes com as formas graves da malária, incluindo os casos que eventualmente morreram durante o acompanhamento, foram coletadas durante a admissão hospitalar. A partir deste ponto, a metodologia empregada variou de acordo com o estudo. No manuscrito II, alterações

genéticas e dosagens plasmáticas na via de metabolização do heme são estudadas. No estudo III, a resposta imune e inflamatória, mensurada por biomarcadores sorológicos, em diferentes grupos de malária vivax, é investigada por uma abordagem analítica por redes. Por fim, o trabalho IV estuda polimorfismos em genes relacionados à resposta imune (*TNF*, *IL6* e *DDX39B*) e suas relações com dosagens de marcadores inflamatórios e desfechos clíncos da malária por *P. vivax*.

Resumo geral da metodologia empregada para a amostragem dos indivíduos estudados nos manuscritos V e VI

Pacientes com uma síndrome febril aguda foram recrutados na Fundação de Medicina Tropical Dr Heitor Vieira Dourado (FMT-HVD), Manaus, entre 2009 a 2011. Os indivíduos foram testados para malária por visualização direta do parasita por gota espessa e os casos positivos foram confirmados por PCR e recrutados aqueles com *P. vivax*. A infecção pela dengue foi diagnosticada pelo PCR em tempo real nos indivíduos com menos de seis dias de febre, e pela mensuração do anticorpo IgM nos pacientes com mais de sete dias de febre. O sorotipo viral foi determinado por PCR em tempo real. Indivíduos com sinais de disfunção orgânica grave e aguda foram classificados como malária grave de acordo com os mais novos critérios estabelecidos pela OMS ou com hiperbilirrubinemia isolada (bilirrubina total >3mg/dL), uma complicação observada em pacientes com complicações decorrentes da malária (WHO, 2010). Foram excluídos todos os pacientes com diagnóstico molecular e microscópico de malária causada por *P.*

falciparum ou coinfecção *P. vivax* e *P. falciparum* (infecção mista), assim como os pacientes com diagnóstico sorológico positivo para hepatites virais (A, B, C e D), HIV ou leptospirose. Também foram excluídos os indivíduos com malaria vivax que desenvolveram hemólise por primaquina (pacientes usando primaquina evoluindo com hemoglobina <10g/dL e contagem de reticulócitos >1,5%, ou aumento dos níveis de bilirrubina indireta apos o uso de primaquina). No total, foram recrutados 72 indivíduos com malária sintomática moderada, 14 com malária e hiperbilirrubinemia isolada, 17 casos graves, 30 coinfectados com dengue e *P. vivax*, bem como 30 pessoas apenas infectadas pelo vírus da dengue. Durante a inclusão dos pacientes foram coletadas lâminas com gota espessa de sangue e amostra de sangue total e soro. As lâminas foram organizadas em caixas específicas e os demais em freezer – 80 °C. Todos os pacientes receberam o tratamento antimalárico apropriado ou sintomatológico, no caso da dengue, e foram seguidos de acordo com rotina ambulatorial da FMT-HVD. A partir deste ponto, as investigações variaram de acordo com o sub-estudo. No estudo V, é avaliado a resposta imune (citocinas e quimiocinas) e os níveis de hepcidina em indivíduos com malária e hiperbilirrubinemia isolada, malaria grave e sintomáticos moderados. No manuscrito VI, por sua vez, é descrita a resposta imunoinflamatória dos casos de coinfecção dengue e *P. vivax*.

5. MANUSCRITOS

5.1 MANUSCRITO I

The Host Genetic Diversity in Malaria Infection

Esta revisão descreve o estado da arte da influência das alterações genéticas no ser humano na evolução clínica da malária.

Resumo dos resultados: alterações genéticas nos eritrócitos, mediadores do sistema imune e nos mecanismos de citoaderência em seres humanos influenciam no desfecho clínico da malária. Alguns estudos sugerem que efeitos combinados de um grupo de fatores genéticos nas hemácias e resposta imune podem ser relevantes em mediar a resistência ou susceptibilidade na infecção pelo *Plasmodium*. Contudo, resultados de estudos em genética precisam ser interpretados com cautela visto que diferenças na frequência e funcionalidade dos polimorfismos podem variar conforme diferentes populações.

Este trabalho foi publicado no periódico internacional *Journal of Tropical Medicine.*

Hindawi Publishing Corporation
Journal of Tropical Medicine
Volume 2012, Article ID 940616, 17 pages
doi:10.1155/2012/940616

Review Article

The Host Genetic Diversity in Malaria Infection

Vitor R. R. de Mendonça,[1,2] Marilda Souza Goncalves,[1,3,4] and Manoel Barral-Netto[1,2,5]

[1] *Laboratório Integrado de Microbiologia e Imunorregulação (LIMI), CPqGM, FIOCRUZ, Rua Waldemar Falcão 121, Candeal, 40296-710 Salvador, BA, Brazil*
[2] *Faculdade de Medicina, Universidade Federal da Bahia, 40296-710 Salvador, BA, Brazil*
[3] *Faculdade de Farmácia, Universidade Federal da Bahia, 40296-710 Salvador, BA, Brazil*
[4] *Instituto de Ciência e Tecnologia do Sangue, Campinas, SP, Brazil*
[5] *Instituto de Investigação em Imunologia, Instituto Nacional de Ciência e Tecnologia, São Paulo, SP, Brazil*

Correspondence should be addressed to Manoel Barral-Netto, manoel.barral@gmail.com

Received 13 August 2012; Revised 6 November 2012; Accepted 19 November 2012

Academic Editor: Christophe Chevillard

Copyright © 2012 Vitor R. R. de Mendonça et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Populations exposed to *Plasmodium* infection develop genetic mechanisms of protection against severe disease. The clinical manifestation of malaria results primarily from the lysis of infected erythrocytes and subsequent immune and inflammatory responses. Herein, we review the genetic alterations associated with erythrocytes or mediators of the immune system, which might influence malaria outcome. Moreover, polymorphisms in genes related to molecules involved in mechanisms of cytoadherence and their influence on malaria pathology are also discussed. The results of some studies have suggested that the combinatorial effects of a set of genetic factors in the erythrocyte-immunology pathway might be relevant to host resistance or susceptibility against *Plasmodium* infection. However, these results must be interpreted with caution because of the differences observed in the functionality and frequency of polymorphisms within different populations. With the recent advances in molecular biology techniques, more robust studies with reliable data have been reported, and the results of these studies have identified individual genetic factors for consideration in preventing severe disease and the individual response to treatment.

1. Introduction

Malaria is one of the most important and prevalent infectious diseases in the world. The World Health Organization (WHO) estimated 225 million malaria cases worldwide with 781,000 deaths due to *Plasmodium* infection per year [1]. Four species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) are responsible for almost all human infections [2].

Malaria has been associated to gene selective pressure in the human genome, and it has been associated as an evolutionary force of some genetic diseases, such as sickle cell disease (SCD), thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and other red blood cell (RBC) genetic anemia with Mendelian inheritance. Haldane (1949) suggested a "balanced polymorphism" where the hemoglobin S (HbS) homozygote disadvantage is recompensed through the resistance of the heterozygote (HbAS) in regions where malaria is endemic [3]. Reports associating several genetic disorders with malaria susceptibility or resistance are on the rise, and studies of heritability indicate that approximately 25% of the risk for severe malaria progression is determined through human genetic factors [4].

Genetic epidemiology may help in pointing out major molecular pathways of some infectious diseases, such as malaria, which involve a robust immune and inflammatory response and the participation of erythrocytes and other blood cells in its pathogenesis. The aim of this paper is to review the major genetic alterations in the human host associated with the clinical spectrum of malaria infection and disease development. We specifically address the areas of inherited disorders in red blood cells (RBC) and mutations in the genes of key molecules during the immune response that confer an increase of susceptibility or resistance against malaria. The multiplication of *Plasmodium* inside the RBC and its subsequent rupture have been implicated in several

phenomena present in the malarial syndrome. A protective effect against malaria infection has been associated with genetic disorders involving the RBC, such as cytoskeleton disorders, surface antigen gene mutations, enzymatic machinery deficiencies, or hemoglobin alterations [5]. The immune response is critical for controlling *Plasmodium* infection, and the balance between proinflammatory (Th1-type) and anti-inflammatory (Th2-type) cytokines has been implicated in both the control of parasite multiplication and the development of symptoms. The genetic background of the affected individual might also influence cytokine expression and disease outcomes [6, 7]. Notably, the frequency of genetic alterations differs depending on the population origin and structure, and some mutations might differentially influence the disease outcome in different patterns.

Understanding the genetic alterations involving RBC disorders and the immune response might provide insight into the development of new strategies for host-genotype treatment and/or the prevention of malaria.

2. Inherited Disorders of Red Blood Cells and Malaria

2.1. Membrane and Enzymatic Disorders of Erythrocytes. Several membrane-inherited disorders of RBC provide additional information concerning the pathogenesis of malaria. Hereditary spherocytosis is a disorder characterized by the loss of membrane lipid surface. This common hemolytic anemia also reflects ineffective integral protein interactions and is associated with lower parasitemia [8]. Other RBC membrane inherited disorders include hereditary ovalocytosis, elliptocytosis, pyropoikilocytosis, and acanthocytosis. Elliptocytosis has been associated to resistance against invasion by *P. falciparum* in humans and *P. knowlesi* in experimental models [9]. However, ovalocytosis is a RBC-inherited cytoskeleton disorder most commonly associated with malaria. A particular type of southeast Asian ovalocytosis (SAO), also known as Melanesian elliptocytosis or stomatocytic elliptocytosis, is characterized by an inherited dominant trait related to heterozygosity for a 27-pair deletion in the gene encoding protein band 3 (*SLC4A1Δ27*) in the erythrocyte membrane [10]. Although SAO homozygosity has been associated with embryo mortality, its heterozygosity is associated with the absence of clinical symptoms and a lack of hemolysis. Ovalocytes are characterized as rigid and more resistant to changes in the shape as a result of low osmotic fragility and the low expression of several RBC antigens [11]. SAO ovalocytes characteristics have been associated with resistance to malaria infection, particularly against *P. falciparum* merozoites invasion [11]. Patients with elliptocytosis exhibit a similar degree of parasitemia independent of disease severity [11–13].

The Duffy, also called Duffy antigen/chemokine receptor (DARC), Fy glycoprotein, or CD234, is a RBC antigen encoded by the *DARC* human gene that is considered to be a nonspecific receptor for several chemokines. The *P. vivax* merozoite uses the Fy antigen to invade RBC [14]. The *Fy* antigen possesses two distinct alleles known as *Fya* and *Fyb*, which result from a single point mutation in codon 42 (rs2814778) that results in a glycine to asparagine substitution within the protein. Another polymorphism (−33T>C, no rs designation available) in the promoter region of the *DARC* gene ablates DARC expression on the surface of erythrocytes. Erythrocytes expressing *Fya* exhibited 41–50% lower binding to *P. vivax* compared with *Fyb*, and individuals with the Fya+b-phenotype showed a 30–80% lower risk of developing clinical symptoms of vivax malaria [15]. The RBC of individuals with the *Fy-33* genotype are not susceptible to *P. vivax* merozoites invasion and are refractory to the erythrocytic stage of the disease. However, the hepatic malaria stage has been observed in these individuals, making them reservoirs for the disease [14]. With regard to *P. falciparum* infection, more than one receptor on the surface of RBC is responsible for merozoite infection, which include glycophorin A, B, and C (GPA, GPB, and GPC), protein band 3 and others (Y receptors, E, Z, and X), whose molecular identity has not yet been determined [16]. The genetic polymorphisms in the *band 3* or *GYPC* gene are highly prevalent in malaria endemic areas (Papua New Guinea) and confer resistance to severe diseases [17]. Polymorphisms identified in other receptors genes, such as *GYPA* and *GYPB*, have been shown to confer only partial protection against the *Plasmodium* invasion of RBC [18, 19]. Furthermore, in the Brazilian Amazon, an SNP in the *GYPB* receptor gene (rs7683365) was associated with host susceptibility to *P. falciparum* infection [17].

The G6PD deficiency and low levels of pyruvate kinase are the most prevalent genetic alterations in RBC that can influence malaria outcomes. G6PD is a metabolic enzyme that catalyzes the first reaction in the pentose phosphate pathway, providing energy for the RBC in the form of nicotinamide adenine dinucleotide phosphate (NADPH). NADPH enables RBC to counterbalance oxidative stress through oxidant agents [20]. The *G6PD* gene is located on the X chromosome and is therefore more prevalent in men [21]. The G6PD deficiency is the most common cause of hereditary hemolytic anemia and is more prone to oxidative stress from the decreased production of NADPH. The clinical picture of G6PD deficiency involves different degrees of disease severity, which might include hemolytic anemia, neonatal hyperbilirubinemia, and asymptomatic cases [22]. Approximately 400 million people living in tropical and subtropical areas exhibit a G6PD deficiency, with a high diversity of variants, including the common *G6PD B* (wild type), *G6PD A* (nondeficient type), and *G6PD A-* (African deficient type) [23]. It has been suggested that G6PD deficient RBC might reduce intracellular parasite growth [24]. Moreover, studies have shown that infected erythrocytes deficient of G6PD were more phagocytosed by monocytes, which might be associated with the reduction of the parasitic load of the disease [24, 25]. Another enzyme associated with energy production is pyruvate kinase (PK), which is also an important factor in the susceptibility to malaria, and its deficiency has been associated with the reduced survival and increased phagocytosis of parasite-infected erythrocytes [26]. PK deficiency is the second most common cause of hereditary nonspherocytic hemolytic anemia in humans [27]. PK catalyzes the rate-limiting step of glycolysis, and the

energy for erythrocytes is derived from glycolysis, as RBC lack mitochondria. The *PK* gene is highly pleomorphic and includes 59 SNPs and several loss-of-function variants that might be associated with decreased resistance to malaria [27].

2.2. Hemoglobin Alterations. Hemoglobin (Hb), the main compound of erythrocytes, is a tetrameric protein that consists of two pairs of unlike globin chains; each globin chain is associated with one prosthetic group, called a heme group. Hemoglobinopathies are inherited disorders of Hb that can be classified into two major groups. The first group involves structural alterations or variants of Hb, such as HbS, HbC, and HbE; the second group of hemoglobinopathies is classified as synthesis defects of Hb and has been associated with a decrease or absence of globin chain synthesis, with the most common alteration related to the alpha and beta-globin chains (alpha- and beta-thalassemia, resp.).

The alpha-thalassemia (alpha-thal) is the most common genetic disorder in the human population and is caused by the decreased or absent synthesis of the alpha globin chain due to the deletion or nondeletion mutation of one or both *alpha-globin* genes (*HBA1* and *HBA2*), located on chromosome 16 [28, 29]. A 3.7-kilobase (Kb) deletion determines the most common form of alpha-thal, also called alpha$^+$-thal, and there is unique potential for a rightward crossover between the *HBA* genes. One study reported that alpha$^+$-thal increased the incidence of mild malaria [5]. However, several subsequent studies reported that alpha$^+$-thal was associated with a reduced risk of uncomplicated malaria episodes [30] or a protective effect against severe forms of malaria [31–35].

Beta-thalassemia is characterized by the decreased synthesis of the beta-globin chain through a genetic alteration in the *beta-globin* gene (*HBB*) of the human *globin* gene cluster located on chromosome 11 [36]. The heterozygote of this inherited trait is associated with a mild anemia and an ineffective erythropoiesis, while the homozygote mutant is associated with severe anemia and the risk of early death. The beta-thalassemia trait is associated with a relative resistance against *P. falciparum* malaria [37] and protection against severe malaria forms [38].

The presence of a single nucleotide mutation (rs334) in the *beta-globin* gene (*HBB*) is associated with a structural modification of the beta polypeptide chain Glu6Val (HbS), resulting in the variant HbS, which can be found in the asymptomatic heterozygous state. This variant is commonly known as the sickle cell trait (HbAS). In 1978, Friedman suggested that the mechanism of resistance against *Plasmodium* of RBC with HbS might be solely due to intraerythrocytic conditions [39]. HbS polymerizes under deoxygenate conditions, and parasites become severely affected; HbS RBC become sickled, with the increased phagocytosis of infected erythrocytes [40, 41]. The presence of HbS in severe malaria patients is associated with less hemolysis and reduced levels of free heme [42, 43]. Many studies have described an association between the heterozygote HbAS and protection against malaria, with more than 90% protection against severe forms [44, 45]. A large genome-wide association (GWA) analysis of severe malaria cases in four different ethnic groups in Gambia has confirmed the role of HbS variant in resistance to malaria [46]. However, this study did not find new genetic associations as a result of the need for population-specific data on genome sequence variation. Therefore, it is difficult to design effective multicenter replication studies without information about sequence variation and haplotype structure in those African populations [46]. Moreover, in another GWA study carried out in Ghana, besides confirmation of previous reports on protective effects of HbS and blood group O, two novel resistance loci were described for severe malaria [47]. One of the loci was identified on chromosome 1q32 within the *ATP2B4* gene, which encodes the main calcium pump of erythrocytes; and the other locus was indicated by an SNP on chromosome 16q22.2, possibly linked to a gene encoding the tight-junction MARVELD3, which may have a role in microvascular damage caused by endothelial adherence of parasitized erythrocytes [47].

Hemoglobin C (HbC) occurs from a point mutation leading to a Glu6Lys substitution at the sixth position in the beta-globin polypeptide chain (rs33930165) [48], and hemoglobin E (HbE) results from a substitution of Glu26Lys at position 26 in the beta-globin polypeptide chain (rs33950507) [49]. HbC homozygotic individuals exhibit mild hemolysis and splenomegaly, while the heterozygotic state is asymptomatic [50]. The RBC from HbE homozygous individuals is microcytic, with low hemoglobin concentrations, which reduces the possibility of merozoite invasion and impairs parasite growth within the variant RBC [51]. The presence of both protective factors, HbC and HbE, has been associated with a lower risk of developing severe forms of malaria [48, 49, 51–54].

Genetic alterations in erythrocytes were probably the first to be discovered as a result of the evolutionary pressure of malaria in the human genome. Mutations affecting several protection mechanisms related to RBC have been described for HbS [55]. Furthermore, several polymorphisms within the membrane cytoskeleton, surface antigen, and enzymatic machinery and other hemoglobin alterations within RBC influence the susceptibility and resistance against malaria.

2.3. Systemic Regulations of Heme. During the erythrocytic stage of malaria, merozoites multiply inside RBC, which result in the rupture of this structure and subsequent release of free Hb into the circulating blood. In the presence of reactive oxygen species (ROS), Hb releases its heme prosthetic group. In addition, the *Plasmodium* is responsible for the degradation of 60% to 80% of the total Hb [56]. Hb degradation contributes to heme release and ROS generation, which are harmful to both erythrocytic schizonts and the host [57]. Free heme is harmful to cells and tissues and can induce oxidative stress, inflammation, cytotoxicity [58], and cell death [59]. The *Plasmodium* has developed a series of protective mechanisms against the deleterious effects of free heme through the polymerization of heme in hemozoin, a malarial pigment that counteracts the pro-oxidative effects of iron (Fe) present in protoporphyrin IX [56]. The host also displays mechanisms of protection against free Hb cytotoxicity. Under homeostatic conditions, free Hb

is released from the intravascular lysis of RBC and rapidly binds to haptoglobin (Hp). The CD163 receptor, expressed on monocytes/macrophages in the red pulp of the spleen, recognizes and internalizes the Hb/Hp complex [60]. Following internalization of the Hb/Hp complex, heme is degraded through the enzymatic action of heme-oxygenase (HO-1), producing biliverdin, iron, and carbon monoxide (CO). The beta chain (β) of Hp is approximately 40 kDa, and the alpha 1 chain (α1), which is synthesized by the allele variant *Hp1*, is approximately 8.86 kDa and is subdivided into *1S* and *1F*; the alpha 2 chain (α2), which is synthesized by the allele variant *Hp2*, is approximately 17.3 kDa [61]. These alleles have different affinities for free Hb (*Hp1.1* > *Hp1.2* > *Hp2.2*) and for CD163 (*Hp2.2* > *Hp1.2* > *Hp1.1*) [60]. The presence of the *Hp2.2* genotype has been associated with increased iron redox activity and oxidative stress compared with the *Hp1.1* genotype [62, 63]. Furthermore, monocytes can internalize the Hb/Hp2.2 complex, but not Hb/Hp1, and stimulate the release of proinflammatory cytokines [64]. Thus, the *Hp2.2* has been associated with an increased susceptibility to various inflammatory conditions, including malaria. We have recently reported that subjects with the *Hp2.2* genotype display a higher risk of developing symptomatic mild malaria (as opposed to asymptomatic) upon *Plasmodium* infection [65].

The induction of HO-1 in experimental models of malaria has been associated with increased resistance to malaria as a result of HO-1 in controlling heme-induced nonspecific tissue damage and inflammation [55, 66]. There is an (GT)n repeat polymorphism microsatellite in the promoter of the *HMOX1* gene, which is associated with the increased or decreased synthesis of HO-1 in response to different stimuli [67]. Individuals with lower (GT)n dinucleotide repeats have a higher expression of HO-1, while higher (GT)n dinucleotide repetition is associated with the decreased synthesis of the protein [67]. This polymorphism has been described in several chronic degenerative diseases [68–73], but its role in malaria is controversial. The results of some studies have shown that the presence of lower (GT)n repeats in *HMOX1* gene is associated with a greater chance of developing severe malaria, suggesting that the increased expression of HO-1 is deleterious for human malaria [74, 75]. We have shown that subjects with the long form (≥30 GT repeats) of the *HMOX1* gene polymorphism have greater susceptibility to developing malaria and higher inflammatory scores than individuals with the short form [65]. However, studies in mice have shown that HO-1 is highly beneficial for malaria by conferring host tolerance to *Plasmodium* infection. Sickle Hb induces the expression of HO-1, which leads to CO production. CO binds with high affinity to free Hb and prevents the release of heme from hemoglobin, which reduces systemic levels of deleterious free heme [55, 66]. Other *HMOX1* gene polymorphisms have been described, which correspond to the single nucleotide polymorphisms −1135G>A (no rs designation available) and −413A>TG (no rs designation available). However, only the last SNP seems to have functional importance [67].

Heme reduces the production of prostaglandin E2 (PGE2) and TGF-β from mononuclear cells through superoxide dismutase-1 (SOD-1), an enzyme responsible for the detoxification of harmful superoxide [76]. SOD-1 is a powerful predictor of malaria severity in individuals infected with *P. vivax* with higher sensitivity and sensibility than TNF-α levels [77], confirming the importance of this enzyme in malaria pathogenesis. The preliminary data from our group showed an association between several SNPs in the *SOD-1* gene and different expressions of this enzyme in subjects with malaria. Furthermore, those *SOD-1* SNPs were associated with malaria symptoms in individuals infected with *P. vivax*, indicating that the genetic predisposition of the individual might alter the response of these subjects against ROS.

3. Immune Response

3.1. Toll-Like Receptors (TLR). Toll-like receptors are a family of transmembrane proteins present in monocytes, macrophages, and dendritic cells, which play a key role in the innate immune response. TLR recognize pathogen associated molecular patterns (PAMPs) through extracellular receptor modules and initiate the inflammatory cascade through the transcription of inflammatory cytokines, type 1 interferon, and chemokines through NF-kB or interferon regulatory factor dependent pathways [78, 79]. Furthermore, the stimulation of TLRs also leads to dendritic cell maturation and the induction of the adaptive immune response [80]. Each TLR has a unique pattern of expression, intracellular localization, and signaling pathway, resulting in different immune responses [81, 82]. The intracellular signaling of TLR is mediated through at least five different adapter proteins, including toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), myeloid differentiation primary response gene 88 (MyD88), and toll-like receptor adaptor molecule 1 (TRIF) [83]. TLR1, 2, 4, 5, 6, and 10 are found on the extracellular surface of cells, whereas TLR3, 7, 8, and 9, each of which is a nucleic acid sensor, are located within the endoplasmic reticulum and cytoplasmic vesicles [84]. In the context of malaria infection, TLR2 and TLR4 have been reported to recognize *P. falciparum* glycosylphosphatidylinositol (GPI), while TLR9 has been reported to recognize *Plasmodium* DNA or the hemozoin pigment [85–87].

Some common SNPs in *TLR* genes are functionally important and affect the recognition of ligands and intracellular signaling. These SNPs have been associated with numerous infectious and parasitic diseases [88]. Several studies have associated *TLR* gene polymorphisms with clinical malaria and parasitemia levels. Two polymorphisms have been described in the 5′ untranslated region (UTR) of the *TLR2* gene, a 22-base pair deletion in the first untranslated exon (Δ22), and an (GT)n dinucleotide repeat in the second intron [89]. Both polymorphisms, the deletion and shorter (GT)n repeats, are associated with reduced TLR2 reporter activity and TLR2 expression [90]. However, only the Δ22 heterozygous genotype was associated with protection from cerebral malaria [91]. Other SNPs of *TLR2* (Arg677Trp, no rs designation available and Arg753Gln, rs5743708) were not identified in the *Plasmodium*-infected population [92]. In the case of the *TLR4* gene, the more frequent genetic

alterations studied were two nonsynonymous cosegregating SNPs (Asp299Gly, rs4986790 and Thr399Ile, rs4986791) that modify the ligand-binding site of the receptor [93]. The *TLR4* Asp299Gly was associated with an increased risk of maternal anemia and infant low-birth weight in pregnant women with malaria [94]. In addition, the risk of severe malaria in children was increased 1.5-fold in the presence of *TLR4* Asp299Gly and 2.6-fold with *TLR4* Thr399Ile [92].

The *TLR9* gene has been associated with the pathogenesis of severe malaria in humans and experimental models. Studies have demonstrated that TLR9-deficient mice survived more during cerebral malaria and that the antagonist-mediated TLR9 inhibition conferred protection against cerebral malaria in mice [95, 96]. A human study analyzing the (rs187084, −1486C>T) polymorphism in the promoter region of *TLR9* gene showed an increased risk of low birth weight among infants from pregnant women with malaria, whereas increased parasitemia was observed in adults with mild malaria [84, 94]. However, in a large family and population-based association study, Malawi and Gambia showed that the effects of the four most common SNPs in the *TLR9* gene, rs187084 (−1486C>T), rs5743836 (1237C>T), rs352139 (1174G>A), and rs352140 (2848G>A), were not associated with severe malaria [97]. Smaller population studies in Brazil, Iran, and Ghana showed associations between these polymorphisms and mild clinical malaria in their respective populations, raising the possibility that the *TLR9* gene polymorphisms might be associated with a milder form of the disease [84, 98, 99]. The TIRAP/Mal interaction with tumor necrosis factor receptor-associated factor 6 (TRAF6) is responsible for mediating the downstream signaling of TLR2 and TLR4 to induce a proinflammatory response [100]. An SNP mutation in the *TIRAP* gene (rs8177374; S180L) has been associated with both protection against malaria [101] and susceptibility to the development of mild malaria [97], but the association of the *TIRAP* gene polymorphism with malaria remains controversial.

The importance of TLR in malaria infection has been recently described, particularly with regard to TLR2, 4, and 9. Genetic alterations in *TLR* and their signaling pathways remain controversial. Thus far, no conclusive evidence of polymorphisms in these receptors that might influence the disease outcome and effect host-genotype treatment have been identified.

3.2. Cytokines. Malaria infection is marked by changes in cytokine expression resulting from individual immune responses. Proinflammatory Th1-type cytokines (IL-1, IL-6, IL-8, IL-12, IFN-gamma, and TNF-α) are critical for controlling the erythrocytic and hepatic stages of *Plasmodium* infection [6, 7], but the excessive production of these cytokines might also contribute to disease manifestations and/or tissue damage, such as the brain in cases of cerebral malaria. It has also been suggested that anti-inflammatory Th2-type cytokines (IL-4, IL-10, and TGF-beta) downregulate Th1-type cytokines and the proinflammatory response, thereby preventing subjects from severe forms of malaria [102]. Furthermore, the new concept of tolerance against diseases has demonstrated protection against malaria. Unlike resistance, tolerance does not affect the pathogen burden but reduces tissue damage and other pathological effects of disease caused by the pathogen or immune response [103]. This tolerance might be due to the genetic profile of the affected individual, and HbS and HO-1 expressions are the most described mechanisms in malaria [55, 66, 104].

TNF-α is a proinflammatory cytokine that has attracted particular interest because of its ambiguous activity in host defense and pathogenesis of cerebral malaria and other serious complications [105]. High concentrations of TNF-α are related to the pathogenesis of symptoms associated with malaria, such as fever, and severe forms of infection, such as cerebral malaria [106, 107]. However, TNF-α has also been associated with the presence of potent antiparasitic activity, and persistent high levels of the cytokine lead to a rapid improvement in fever and a reduction in parasitemia [108, 109]. Genetic alterations in the *TNF* gene have been described in several studies with different populations in the world and sometimes with contradictory results. Population differences in susceptibility or resistance to malaria according to *TNF* SNPs may be a result of diverse evolutionary pressure between ethnicities, as well as different parasite strains and incidence of severe forms of disease. In Gambia, the SNPs *TNF* −308G>A (rs1800629) and *TNF* −238G>A (rs361525) were associated with an increased risk of cerebral malaria and severe malarial anemia, respectively [110, 111]. Studies in Gabon associated the *TNF* −308G>A polymorphism with a shorter interval to malaria reinfection and the *TNF* −238G>A polymorphism with protection against mild symptomatic malaria [23, 112]. In Sri Lanka, the *TNF* −308A allele was associated with severe malaria and other infections [113]. In another study in Mynamar, the *TNFPD* allele haplotype (−238G; −308G; −857T, rs1799724; −1031T, rs1799964) was associated with increased susceptibility to cerebral malaria because the transcription factor OCT-1 binds to *TNF* −857T in the *TNFPD* allele but not to *TNF* −857C in the *TNFPA*, *B* and *C* alleles and interacts with the proinflammatory NF-κB subunit transcription factor p65 at the adjacent binding site [114]. Other studies have shown no association between *TNF* gene polymorphisms and severe malaria in Kenya, Malawi, Mali, Tanzania, and Indonesia [111, 115–117]. It has been shown that lymphotoxin-a (LTa), which belongs to the TNF family, plays an important role in malaria [116]. Since LTa binds to the TNF receptors TNF-R1 and TNF-R2, TNF and LTa may exert their effects via the same receptors. *LTA* polymorphisms may influence resistance to malaria in humans, and two SNPs have been described: *LTA* C+80A (rs2239704) and *LTA* A+252G (rs909253). The first is an SNP that allows specific binding of the transcriptional repressor ABF-1 and, therefore, considered to be a low LTa-producing allele, has been associated with lower *P. falciparum* parasitemia in malaria-endemic Burkina Faso but was not associated with severe malaria in Gambia [118–120]. The SNP rs909253 has been reported to influence LTa production [121], but it was not associated with severe malaria in Sri Lanka [113]. Both SNPs (rs2239704 and rs909253) were reported to not be associated with severe malaria in a study from Gambia, Kenya, Malawi, and Indonesia [111, 116].

The chromosomal region 5q31–33 contains several important genes encoding molecules, such as cytokines, growth factors, and growth factor receptors, which are involved in immunity against *Plasmodium* infection [122]. The 5q31–33 region contains genes encoding cytokines IL-3, IL-4, IL-5, IL-9, IL-12B, IL13, and other genes, such as the *immunologically active interferon regulatory factor-1* [123]. Concerning the genetic control of blood infection levels, linkage analyses studies have demonstrated the involvement of the 5q31–q33 region with parasitemia in populations from Cameroon and Burkina Faso [124, 125]. Asymptomatic parasite density was also linked to chromosome 5q31 in a study from Senegal [126]. A genomewide linkage study revealed three strongly suggestive lines of evidence for linkage between mild malaria attacking both the 6p25.1 and the 12q22 regions and between the 20p11q11 region and the prevalence of parasite density in asymptomatic Senegalese children [127]. Furthermore, in this study, one gene associated with malaria infection in the 5q31–q33 was also detected, confirming the importance of this genetic region in the susceptibility to malaria infection [127].

Type 1 helper T lymphocytes may be protective through the release of IFN-γ, which activates macrophages to destroy parasitized erythrocytes, promotes the production of opsonizing antibodies, and helps to destroy parasites during the hepatic cycle [128]. However, IFN-γ also has proinflammatory effects that may contribute to disease severity [129, 130]. Studies have reported associations between *IFNG* gene polymorphisms and susceptibility to disease. The first intron of the *IFNG* gene contains a highly polymorphic CA-repeat microsatellite, whose 12 CA-repeat allele is associated with high levels of IFN-γ production in vitro [131], and it has been associated with an SNP allele *IFNG*+874T (rs62559044), which coincides with a putative NF-$\kappa\beta$ binding site [132]. In Gambia, no evidence of a strong association between severe malaria and the 12 CA-repeat allele and *IFNG*+874 (rs62559044) polymorphism was observed [133]. However, 14 CA repeats (*IFNG* CA14) were associated with CM in *P. falciparum*-infected children, and *IFNG* −183G/T (no rs designation available) and *IFNG*(CA)14/(CA)14 genotypes were more frequent in children with uncomplicated malaria than in children with cerebral malaria from Mali [134].

Concerning *IL-13*, an SNP −1055T>C (rs1800925) has showed a significant association with protection from severe malaria in Thailand [135]. A fine association mapping in the *IL-13* gene using the same malaria subjects revealed that only rs1881457 located in the promoter region, which is in linkage disequilibrium with rs1800925, showed a significant association with severe malaria [123]. Furthermore, two SNPs (rs848, rs1881457) in *IL-13* gene were found to be significantly different between those who have experienced one or more malaria attacks within past 10 years and those who did not in Sri Lanka [136].

IL-12 is a proinflammatory cytokine that boosts erythropoietic responses in infections with *Plasmodium* parasites. Low levels of IL-12 have been associated with the pathogenesis of malaria in children and nonimmune adults through the promotion of IFN-γ release from cells of the innate immune system, while high levels of this cytokine are associated with severe malaria [137]. IL-12 cytokine is a dimer composed of a 35-kD subunit encoded by the *IL12A* gene (chromosome 3p12-q13.2) and a 40-kD subunit encoded by the *IL12B* gene (chromosome 5q31–33), which exerts its effects on the immune response through receptors encoded by *IL12RB1* and *IL12RB2* [138]. A mutation in the promoter region of *IL12B*, *IL12B-pro* (rs17860508) has been associated with susceptibility to cerebral malaria [139, 140]. This polymorphism has been shown to affect gene expression and the production of cytokines and nitric oxide (4 bp less) [141]. Moreover, polymorphisms in *IL12A* (rs2243140) and *IL12RB1* (rs429774) confer protection against severe malarial anemia [138].

IL-4 is a pleiotropic cytokine with multiple immune-modulating functions in several cells [142]. IL-4 plays an important role in IgE antibody antimalarial responses and regulates the differentiation of precursor T-helper cells into the Th2 subsets that regulate humoral immunity [122, 143]. Several polymorphisms in the *IL-4* gene have been described, and four polymorphisms were described in the promoter region in association with total IgE production [144–146]. Despite the *IL-4* −589C>T (rs2243250) influence on IgE levels, there was no association with severe malaria [122, 147]. However, a recent study that assesses the influence of 11 polymorphism in *IL4* gene on predisposition to malaria in Mali found a genetic association between *IL4* VNTR (rs8179190) and others *IL4* mutations (−33C/T; rs2243267; rs2243268; rs2243282) with severe disease, supporting the view that *IL4* genetic alterations could be a risk factor for malaria severity [129].

IL-1 is an endogenous pyrogen that plays an important role in the innate immune response of the human host to *Plasmodium* infection [148]. Two different genes (*IL1A* and *IL1B*) encode IL-1, which are located in chromosomal region 2q14, an area that also contains genes for IL-1 receptor types 1 and 2 (*ILR1* and *IL1R2*), the IL-1 receptor antagonist (*IL1RN*), and other homologous genes that have not been well characterized [149]. The rapid induction of IL-1β might help control invading malaria parasites through the induction of an acute inflammatory response as part of the first line of defense; however, the overproduction of IL-1β might cause severe pathogenic effects [150]. Three different SNPs in the promoter region of the *IL1B* gene (−3737G>A, no rs designation available; −1464G>C, no rs designation available; −511A>G, rs16944) have been associated with IL-1β plasma levels [151]. The *IL1B* −511A allele was associated with an increased risk of severe malarial anemia and reduced levels of IL-1β [123]. In another study conducted in Gambia, significant associations between variations in *IL1A* +4845G>T (rs17561) and *IL1B* +3954C>T (rs1143634) were associated with symptomatic malaria [148].

IL-10 is an anti-inflammatory Th2-type cytokine produced primarily by monocytes and lymphocytes, and IL-10 exhibits various effects in the regulation of the immune response, including downregulating the expression of the proinflammatory (type 1) immune response [152]. The *IL-10* gene is located on chromosome 1q31-32 within the promoter region and includes the well-defined SNPs

IL-10 −1082A>G (rs1800870), *IL-10* −819T>C (rs1800871), and *IL-10* −592A>C (rs1800872) [153]. The SNP haplotype was associated with susceptibility to severe malarial anemia and functional changes in the plasma concentrations of IL-10, TNF-α, and IL-12 [154]. However, other studies have shown no evidence of association between the polymorphisms in the *IL-10* gene and malaria severity [155]. A study in Gambia showed an association between the haplotype of five SNPs (+4949G, rs3024500/+919C, rs1518110/−627G, rs1800872/−1117C, rs1800896/−3585T, rs1800890) and resistance to cerebral malaria and severe anemia [156].

Several SNPs influence the levels of pro- and anti-inflammatory cytokines in malaria infection and might lead to an imbalance between these molecules that favor increased host susceptibility to *Plasmodium*. Thus, polymorphisms in the immune response might influence host disease tolerance against malaria.

3.3. Immunoglobulin Receptors and Nitric Oxide (NO). Receptors for the Fc fragment of IgG (FcγRs) provide an important link between humoral and cellular immune responses. There are three families of FcγR (I, II, and III). The primary function of FcgRs is the activation of accessory cells against pathogens; thus, FcgRs are essential molecules in the host defense against infection [157]. Among the three classes of FcgR (FcgRI, FcgRII, and FcgRIII), the low-affinity FcgRII class is the most broadly distributed [158]. The *FcγRIIA* gene contains an important SNP with a G>A substitution in the region responsible for encoding the ligand-binding domain in which histidine (H) replaces arginine (R) at position 131 in the extracellular domain (no rs designation available). Both allotypes bind to human IgG1 and IgG3, but the *FcγRIIA H131* allotype exhibits higher binding affinity to the IgG2 and IgG3 than the *FcγRIIA R131* allotype, but none effectively binds to IgG4 [159]. The *FcγRIIA H131* allotype is the only FcγR that binds with high affinity to IgG2, and this allele is essential for the phagocytosis of microorganisms opsonized with IgG2 and the clearance of immune complexes containing IgG2 [160, 161]. Furthermore, a protective role for IgG2 in malaria infections has been described, which involves the activation of immune effector cells through FcγRII [162]. The *RR131* genotype protects against high levels of parasitemia, whereas the *HH131* genotype was associated with susceptibility to severe malaria with high parasite burden [158, 163, 164]. An additional study showed an association between the *FcgRIIA-RR131* genotype and severe malaria [165].

NO is a highly diffusible, lipid soluble-free radical that mediates the resistance of host severe malaria and other diseases. The production of NO and the cellular expression of enzyme-inducible nitric oxide synthase (NOS2) are associated with protection against severe forms of malaria [166]. The protective effect of NO against *Plasmodium* reflects parasite killing through reactive nitrogen metabolites and a decrease of endothelial adhesion molecules [167, 168]. In humans, NO is produced through the enzymatic conversion of L-arginine to L-citrulline using three different NO synthases (NOS), and NOS2 is induced through the response to pathogens and proinflammatory cytokines [166]. Several polymorphisms in the *NOS2* gene have been associated with malaria severity. In Gambia, the SNP *NOS2* −954G> C (no rs designation available) has been associated with resistance to severe malaria [169], whereas in another group of Gambian subjects, short forms of the polymorphic microsatellite (CCTTT) in the *NOS2* transcription start site were associated with fatal malaria [170]. However, in Tanzania, neither *NOS2* −954G>C polymorphisms nor CCTTT repeats were associated with severe malaria [171]. Another SNP in *NOS2*, −1173C>T (no rs designation available), was associated with protection against cerebral malaria in children in Tanzania and severe anemia in malaria individuals of Kenya [172]. However, no association between *NOS2* polymorphisms and susceptibility to malaria was described [166].

The FcgR receptor and NO are important molecules involved in malaria outcomes, and several studies have attempted to associate mutations in these genes with increased susceptibility to develop severe forms of malaria. However, the results are conflicting, and no conclusion has yet been determined.

4. Mechanisms of Cytoadherence

One of the peculiar characteristics of *P. falciparum*-mediated malaria is the adhesion of infected erythrocytes to capillary endothelium [173]. This association contributes to the pathology of falciparum malaria because it causes microvascular occlusion and inhibits the immune response against parasites [174, 175]. This adhesion is one of the possible mechanisms underlying the pathogenesis of severe forms of malaria, such as cerebral and placental malaria [176–178]. The adhesion molecules include intercellular adhesion molecule 1 (ICAM-1, CD54), platelet/endothelial cell adhesion molecule1 (PECAM-1, CD31), vascular cell adhesion molecule1 (VCAM-1), thrombospondin, E-selectin, P-selectin, CD36, and chondroitin sulfate A [179]. Another characteristic of the virulent phenotype that contributes to the pathogenesis of *P. falciparum* is its ability to form rosettes, a property in which parasitized erythrocytes bind uninfected erythrocytes to form clumps of these cells [180]. The mechanism responsible for the virulence of the rosettes includes the microvasculature obstruction of the bloodstream in high parasitemia, favoring the invasion of merozoites and immune evasion [181–183]. The process of rosetting is mediated through ligand binding of *P. falciparum* to erythrocyte membrane protein1 (PfEMP1), which is expressed on the membrane of an infected RBC among a variety of uninfected RBC receptors, such as serum components, blood group antigens A and B, glucosaminoglycans, and complement receptor 1 (CR1) [184].

Autopsy studies of patients with fatal cerebral malaria or severe malarial anemia showed the sequestration of erythrocytes infected with *Plasmodium* on brain vascular endothelial cells with the increased expression of adhesion molecules, particularly ICAM-1 [185]. The results of a study in Killifi (Kenya) confirmed this association, showing that the adhesion of infected erythrocytes was highest in cerebral malaria compared with the asymptomatic control

group [186]. The ICAM-1 (CD54) is a member of the immunoglobulin super-family, and its role in malaria susceptibility is not limited to an interaction with PfEMP1 [187]. ICAM-1 binds lymphocyte function-associated antigen (LFA)-1, facilitating the movement of leukocytes and active nature killer cells beyond the blood brain barrier during *P. falciparum* infection [188, 189]. Two SNPs have been described in the *ICAM-1* gene, *ICAM-1 Killifi* (rs5491), which results from substitution of lysine for methionine at position 56 of the coding sequence, and a less well-described polymorphism (rs5498, K469E) [187]. The *ICAM-1 Killifi* polymorphism has been associated with both the resistance [190, 191] and susceptibility to severe forms of malaria [192]. However, subsequent studies in Gambia, Thailand, Senegal, Nigeria, and Kenya have reported no significant association between malaria phenotypes and either the ICAM-1Kilifi or the SNP identified in exon 6 (rs5498) [187, 193–197].

Most *P. falciparum* antigens bind to the CD36 molecule, and thus CD36 is considered the most important endothelial receptor for the sequestration of infected erythrocytes [186]. CD36 is an 88-kDa glycoprotein expressed on endothelial cells, macrophages, and dendritic cells, among others. However, in contrast to ICAM-1, this molecule is not expressed on the endothelial cells of brain capillaries [198]. CD36 serves as a receptor for several ligands, including low-density lipoprotein cholesterol (LDL-C), collagen, thrombospodin, and anionic phospholipids and participates in macrophage fusion induced through IL-4 [199]. Mutations in the *CD36 receptor* gene have been associated with protection against or susceptibility to severe forms of malaria. The CD36 deficiency might be induced through the two SNPs in the *CD36* gene (T1264G in exon 10, rs3211938 and G1439C in exon 12, no rs designation available), which encode the truncated proteins that were expressed at high frequency in patients with severe malaria in Gambian, Tanzanian, and Kenyan patients [199–201]. This association was confirmed in a study in India, showing an association of the presence of the mutant allele in heterozygous individuals (1264T>G in exon 10) with protection against severe malaria [202]. A screening of *CD36* gene in malaria patients from Thailand identified two SNPs in the promoter region (−14T>C and −53G>T, no rs designation available) that were associated with protection against cerebral malaria and one microsatellite polymorphism in intron 3 with 12 TG repeats that has been associated with the lower risk of cerebral malaria [203]. Genetic alterations in the *CD36* gene influence the malaria outcome, regardless of different conclusions concerning the polymorphisms identified in this molecule, perhaps reflecting differences among the populations and clinical spectrums of the disease.

Platelet-endothelial cell adhesion molecule 1 (PECAM-1/CD31) is expressed in hematopoietic and endothelial cells. This adhesion molecule was identified as an endothelial receptor for erythrocytes infected with *P. falciparum* [204]. The *PECAM-1* gene is polymorphic, and several polymorphisms have previously been described in the extracellular domain (exons three rs668 L/V, exon 8 rs12953 S/N, rs1131012 exon 12 R/G) and promoter region (GATA-2) [205]. Homozygous individuals with L125V and S563N SNPs in the *CD31* gene were associated with an increased risk for developing cerebral malaria in Thailand [206]. However, in Kenya and Papua New Guinea, no association of the L125V SNP with malaria was observed [205]. Furthermore, an SNP in the *PECAM-1* gene (exon 3 rs668 L/V) was identified as a risk factor for malaria in an endemic region, but this gene exhibited a significant association with protection from disease in a nonendemic region [202]. The mutation in exon 3 of the *CD31* gene might affect the regulation of inflammation because it is present within the first IgG-like domain of the PECAM-1 molecule, which has been associated with hemophilic adhesion and regulates leukocyte transmigration [207, 208]. Thus, despite the influence of genetic alterations in the levels of adhesion molecules, polymorphisms might also alter molecular protein structure and impair the binding affinity of other molecules involved in the immunopathogenesis of malaria.

Complement receptor type 1 (CR1/CD35) is a membrane glycoprotein expressed in various cells, including erythrocytes, monocytes, B and T cells, monocytes, and dendritic cells [209]. CR1 binds with high affinity to C3b and C4b components and plays an important role in the clearance of immune complexes [210]. CR1 also plays a role in opsonization and the control of complement activation [209]. The expression of CR1 on erythrocytes has been related to the formation of rosettes, a phenomenon that results from the adhesion of PfEMP 1 on the surface of infected erythrocytes with a variety of membrane receptors on noninfected erythrocytes [180, 211, 212]. This process contributes to the pathogenesis of severe malaria because it causes the obstruction of cerebral capillaries and increases susceptibility to severe malaria anemia [186, 213]. Furthermore, erythrocyte CR1 binds immune complexes in the bloodstream through a process of "immune adherence" and removes them through phagocyte capitation in the liver and spleen [214]. Subjects with high levels of CR1 on erythrocytes are more likely to form rosettes and contribute to the sequestration of cell clumps in the microvasculature of the brain and other vital organs [180]. Moreover, high levels of CR1 also carry immune complex, which might be recognized by monocytes and endothelial cells to produce proinflammatory mediators [184]. The levels of CR1 on erythrocytes are genetically determined and correlated with the HindIII restriction fragment length polymorphism (RFLP) mutation in the *CR1* gene. Homozygous subjects for the 7.4 kb *HindIII* genomic fragment (the *H* allele) have high levels of CR1 on erythrocytes, whereas homozygous individuals for the 6.9-kb genomic *HindIII* fragment (the *L* allele) exhibit low expression, and *HL* heterozygous individuals show intermediary levels of CR1 in the membrane of erythrocytes [211]. The association of this polymorphism with the susceptibility or resistance to malaria is contradictory. In Gambia and other African populations, a significant association between the *L* allele and protection from severe malaria was not observed [193, 215]. In Thailand, the *LL* genotype was demonstrated as a risk factor for severe malaria [216], and in Papua New Guinea, individuals heterozygous for the *L* allele (*HL*) were correlated with protection against severe malaria [217]. A new polymorphism in the promoter region of the

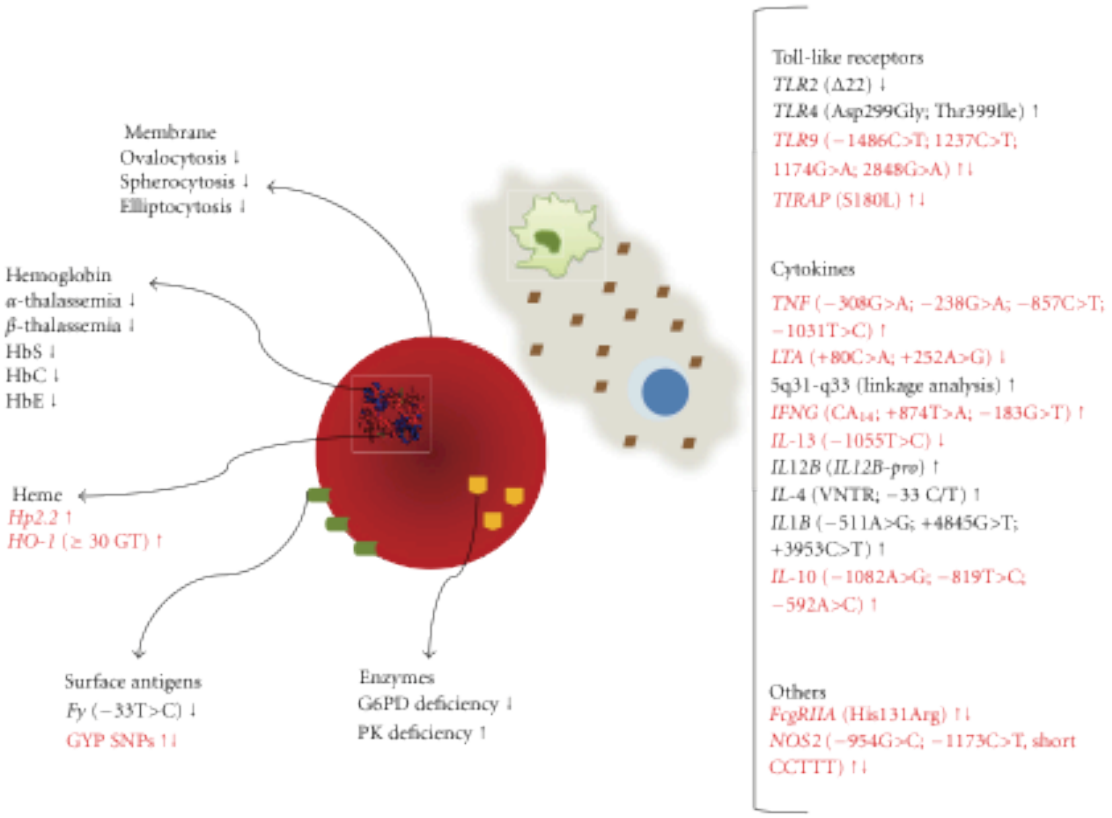


Figure 1: Influence of erythrocyte and immune response gene polymorphisms in malaria outcome. The diagram summarizes the major genetic alterations identified in the erythrocyte and immune response pathways that influence malaria outcome. The up arrow indicates susceptibility, and the down arrow indicates resistance to malaria. Contradictory or not confirmed results are represented by red font color. The protective effect of inherited genetic disorders involving the RBC on malaria infection has been associated with membrane cytoskeleton disorders, surface antigen mutations, enzymatic machinery deficiencies, or hemoglobin and its heme prosthetic group alterations. Considering the immune response, several polymorphisms in gene encoding the TLR receptors and in important cytokines involved in malaria immunopathology are described in the literature. Furthermore, genetic alterations in the FcgRIIA receptor and nitric oxide synthase were also associated with resistance/susceptibility to malaria.

CR1 gene (rs9429942) was associated with higher levels of CR1 on the surface of RBC and protection against cerebral malaria in Thailand [218]. Different associations between the *CR1* genotype with malaria might be associated with the endemicity of malaria in different regions and an under or overestimation of the actual CR1 levels and interactions between *CR1* and other genetic alterations [184].

5. Conclusion

Over the past several years, an increase in the number of scientific publications associated with the genetic predisposition to malaria and severe forms of this disease has been observed. As a result of technological advances, studies of SNPs were exchanged for studies with sophisticated gene sequencing and analyses using advanced molecular biology software. On the basis of the discovery of new functional mutations that alter the expression of several proteins fundamentally implicated in malaria pathogenesis, it is possible to individualize patient care depending on host genotype, as previously demonstrated [219]. However, molecular epidemiology studies should always be interpreted with caution because of the differences in the functionality and frequency of the polymorphisms observed in different populations as a result of diverse evolutionary pressure between different ethnicities.

The clinical manifestation of malaria is primarily described by the lysis of infected erythrocytes and subsequent immune and inflammatory response. Thus, it is critical to understand the role of genetic alterations in this pathway that might influence the disease outcome and severity of malaria. Furthermore, it should be observed that not merely one genetic alteration but rather the combination of a set of genetic factors might influence the susceptibility or resistance to malaria (Figure 1). The results from research studies have already shown that individual genetic factors must be considered for the prevention from severe diseases and individual responses to treatment.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

The authors would like to thank the Instituto Nacional de Ciência e Tecnologia (INCT) for financial support. The authors also thank Jorge Tolentino, Marcio Santana, and Adorielze Leite for technical and logistical support. The work from our laboratory reviewed herein was supported through grants from FINEP, CNPq, and FAPESB. M. S. Goncalves and M. Barral-Netto are senior investigators from CNPq.

References

[1] "WHO—World Health Organization," http://www.who.int/malaria/en/.

[2] M. T. Cavasini, W. L. Ribeiro, F. Kawamoto, and M. U. Ferreira, "How prevalent is *Plasmodium malariae* in Rondônia, western Brazilian Amazon?" *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 33, no. 5, pp. 489–492, 2000.

[3] J. B. S. Haldane, "Suggestions as to quantitative measurement of rates of evolution," *Evolution*, vol. 3, no. 1, pp. 51–56, 1949.

[4] M. J. Mackinnon, T. W. Mwangi, R. W. Snow, K. Marsh, and T. N. Williams, "Heritability of malaria in Africa," *PLoS Medicine*, vol. 2, no. 12, article e340, 2005.

[5] T. N. Williams, K. Maitland, S. Bennett et al., "High incidence of malaria in α-thalassaemic children," *Nature*, vol. 383, no. 6600, pp. 522–525, 1996.

[6] L. Schofield, J. Villaquiran, A. Ferreira, H. Schellekens, R. Nussenzweig, and V. Nussenzweig, "γ Interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites," *Nature*, vol. 330, no. 6149, pp. 664–666, 1987.

[7] S. G. S. Vreden, M. F. van den Broek, M. C. Oettinger, J. P. Verhave, J. H. E. T. Meuwissen, and R. W. Sauerwein, "Cytokines inhibit the development of liver schizonts of the malaria parasite plasmodium berghei in vivo," *European Journal of Immunology*, vol. 22, no. 9, pp. 2271–2275, 1992.

[8] S. Schulman, E. F. Roth, B. Cheng et al., "Growth of *Plasmodium falciparum* in human erythrocytes containing abnormal membrane proteins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 18, pp. 7339–7343, 1990.

[9] T. J. Hadley and L. H. Miller, "Invasion of erythrocytes by malaria parasites: erythrocyte ligands and parasite receptors," *Progress in Allergy*, vol. 41, pp. 49–71, 1988.

[10] T. N. Williams, "Human red blood cell polymorphisms and malaria," *Current Opinion in Microbiology*, vol. 9, no. 4, pp. 388–394, 2006.

[11] C. Kidson, G. Lamont, A. Saul, and G. T. Nurse, "Ovalocytic erythrocytes from Melanesians are resistant to invasion by malaria parasites in culture," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 78, no. 9, pp. 5829–5832, 1981.

[12] P. G. Gallagher, "Red cell membrane disorders," *Hematology*, pp. 13–18, 2005.

[13] B. Genton, F. Al-Yaman, C. S. Mgone et al., "Ovalocytosis and cerebral malaria," *Nature*, vol. 378, no. 6557, pp. 564–565, 1995.

[14] A. Maestre, C. Muskus, V. Duque et al., "Acquired antibody responses against *Plasmodium vivax* infection vary with host genotype for duffy antigen receptor for chemokines (DARC)," *PLoS ONE*, vol. 5, no. 7, Article ID e11437, 2010.

[15] C. L. King, J. H. Adams, J. Xianli et al., "Fy(a)/Fy(b) antigen polymorphism in human erythrocyte Duffy antigen affects susceptibility to*Plasmodium vivax* malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 50, pp. 20113–20118, 2011.

[16] A. F. Cowman and B. S. Crabb, "Invasion of red blood cells by malaria parasites," *Cell*, vol. 124, no. 4, pp. 755–766, 2006.

[17] E. Tarazona-Santos, L. Castilho, D. R. T. Amaral et al., "Population genetics of GYPB and association study between GYPB∗S/s polymorphism and susceptibility to *P. falciparum* infection in the Brazilian Amazon," *PLoS ONE*, vol. 6, no. 1, Article ID e16123, 2011.

[18] D. J. Weatherall, "Host genetics and infectious disease," *Parasitology*, vol. 112, supplement, pp. S23–S29, 1996.

[19] L. H. Miller, "Impact of malaria on genetic polymorphism and genetic diseases in Africans and African Americans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 7, pp. 2415–2419, 1994.

[20] P. Phompradit, J. Kuesap, W. Chaijaroenkul et al., "Prevalence and distribution of glucose-6-phosphate dehydrogenase (G6PD) variants in Thai and Burmese populations in malaria endemic areas of Thailand," *Malaria journal*, vol. 10, article 368, 2011.

[21] M. D. Cappellini and G. Fiorelli, "Glucose-6-phosphate dehydrogenase deficiency," *The Lancet*, vol. 371, no. 9606, pp. 64–74, 2008.

[22] J. E. Frank, "Diagnosis and management of G6PD deficiency," *American Family Physician*, vol. 72, no. 7, pp. 1277–1282, 2005.

[23] L.-E. Mombo, F. Ntoumi, C. Bisseye et al., "Human genetic polymorphisms and asymptomatic *Plasmodium falciparum* malaria in Gabonese schoolchildren," *American Journal of Tropical Medicine and Hygiene*, vol. 68, no. 2, pp. 186–190, 2003.

[24] M. Cappadoro, G. Giribaldi, E. O'Brien et al., "Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by *Plasmodium falciparum* may explain malaria protection in G6PD deficiency," *Blood*, vol. 92, no. 7, pp. 2527–2534, 1998.

[25] K. Ayi, F. Turrini, A. Piga, and P. Arese, "Enhanced phagocytosis of ring-parasitized mutant erythrocytes: a common mechanism that may explain protection against falciparum malaria in sickle trait and beta-thalassemia trait," *Blood*, vol. 104, no. 10, pp. 3364–3371, 2004.

[26] K. Ayi, G. Min-Oo, L. Serghides et al., "Pyruvate kinase deficiency and malaria," *The New England Journal of Medicine*, vol. 358, no. 17, pp. 1805–1810, 2008.

[27] J. Berghout, S. Higgins, C. Loucoubar, A. Sakuntabhai, K. C. Kain, and P. Gros, "Genetic diversity in human erythrocyte pyruvate kinase," *Genes and Immunity*, vol. 13, no. 1, pp. 98–102, 2012.

[28] Y. Yuthavong and P. Wilairat, "Protection against malaria by thalassaemia and haemoglobin variants," *Parasitology Today*, vol. 9, no. 7, pp. 241–245, 1993.

[29] D. J. Weatherall, "Thalassaemia and malaria, revisited," *Annals of Tropical Medicine and Parasitology*, vol. 91, no. 7, pp. 885–890, 1997.

[30] A. Enevold, J. P. Lusingu, B. Mmbando et al., "Reduced risk of uncomplicated malaria episodes in children with alpha +-thalassemia in Northeastern Tanzania," *American Journal of Tropical Medicine and Hygiene*, vol. 78, no. 5, pp. 714–720, 2008.

[31] S. J. Allen, A. O'Donnell, N. D. E. Alexander et al., "α+-Thalassemia protects children against disease caused by other infections as well as malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 26, pp. 14736–14741, 1997.

[32] S. Wambua, T. W. Mwangi, M. Kortok et al., "The effect of α+-thalassaemia on the incidence of malaria and other diseases in children living on the coast of Kenya," *PLoS Medicine*, vol. 3, no. 5, article e158, 2006.

[33] T. N. Williams, S. Wambua, S. Uyoga et al., "Both heterozygous and homozygous α+ thalassemias protect against severe and fatal *Plasmodium falciparum* malaria on the coast of Kenya," *Blood*, vol. 106, no. 1, pp. 368–371, 2005.

[34] F. P. Mockenhaupt, S. Ehrhardt, S. Gellert et al., "α+-thalassemia protects African children from severe malaria," *Blood*, vol. 104, no. 7, pp. 2003–2006, 2004.

[35] J.-B. Gahutu, A. Musemakweri, G. Harms, and F. P. Mockenhaupt, "Prevalence of classic erythrocyte polymorphisms among 749 children in southern highland Rwanda," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 106, no. 1, pp. 63–65, 2012.

[36] S. K. Das and G. Talukder, "A review on the origin and spread of deleterious mutants of the β-globin gene in Indian populations," *Journal of Comparative Human Biology*, vol. 52, no. 2, pp. 93–109, 2001.

[37] M. Willcox, A. Bjorkman, and J. Brohult, "A case-control study in northern Liberia of *Plasmodium falciparum* malaria in haemoglobin S and β-thalassaemia traits," *Annals of Tropical Medicine and Parasitology*, vol. 77, no. 3, pp. 239–246, 1983.

[38] G. A. Luzzi, A. H. Merry, C. I. Newbold, K. Marsh, G. Pasvol, and D. J. Weatherall, "Surface antigen expression on *Plasmodium falciparum*-infected erythrocytes is modified in α- and β-thalassemia," *The Journal of Experimental Medicine*, vol. 173, no. 4, pp. 785–791, 1991.

[39] M. J. Friedman, "Erythrocytic mechanism of sickle cell resistance to malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 75, no. 4, pp. 1994–1997, 1978.

[40] Y. A. Abu-Zeid, N. H. Abdulhadi, L. Hviid et al., "Lymphoproliferative responses to *Plasmodium falciparum* antigens in children with and without the sickle cell trait," *Scandinavian Journal of Immunology*, vol. 34, no. 2, pp. 237–242, 1991.

[41] D. J. Weatherall, "Common genetic disorders of the red cell and the 'malaria hypothesis'," *Annals of Tropical Medicine and Parasitology*, vol. 81, no. 5, pp. 539–548, 1987.

[42] J. Balla, H. S. Jacob, G. Balla, K. Nath, J. W. Eaton, and G. M. Vercellotti, "Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 20, pp. 9285–9289, 1993.

[43] J. Balla, H. S. Jacob, G. Balla, K. Nath, and G. M. Vercellotti, "Endothelial cell heme oxygenase and ferritin induction by heme proteins: a possible mechanism limiting shock damage," *Transactions of the Association of American Physicians*, vol. 105, pp. 1–6, 1992.

[44] C. López, C. Saravia, A. Gomez, J. Hoebeke, and M. A. Patarroyo, "Mechanisms of genetically-based resistance to malaria," *Gene*, vol. 467, no. 1-2, pp. 1–12, 2010.

[45] A. V. S. Hill, C. E. M. Allsopp, D. Kwiatkowski et al., "Common West African HLA antigens are associated with protection from severe malaria," *Nature*, vol. 352, no. 6336, pp. 595–600, 1991.

[46] M. Jallow, K. A. Koram, Y. T. Ying et al., "Genome-wide and fine-resolution association analysis of malaria in West Africa," *Nature Genetics*, vol. 41, no. 6, pp. 657–665, 2009.

[47] C. Timmann, T. Thye, M. Vens et al., "Genome-wide association study indicates two novel resistance loci for severe malaria," *Nature*, vol. 489, no. 7416, pp. 443–446, 2012.

[48] A. Agarwal, A. Guindo, Y. Cissoko et al., "Hemoglobin C associated with protection from severe malaria in the Dogon of Mali, a West African population with a low prevalence of hemoglobin S," *Blood*, vol. 96, no. 7, pp. 2358–2363, 2000.

[49] R. L. Nagel, C. Raventos-Suarez, and M. E. Rabry, "Impairment of the growth of *Plasmodium falciparum* in HbEE erythrocytes," *Journal of Clinical Investigation*, vol. 68, no. 1, pp. 303–305, 1981.

[50] F. P. Mockenhaupt, S. Ehrhardt, J. P. Cramer et al., "Hemoglobin C and resistance to severe malaria in Ghanaian children," *Journal of Infectious Diseases*, vol. 190, no. 5, pp. 1006–1009, 2004.

[51] K. Chotivanich, R. Udomsangpetch, K. Pattanapanyasat et al., "Hemoglobin E: a balanced polymorphism protective against high parasitemias and thus severe P falciparum malaria," *Blood*, vol. 100, no. 4, pp. 1172–1176, 2002.

[52] D. Modiano, G. Bancone, B. M. Ciminelli et al., "Haemoglobin S and haemoglobin C: "Quick but costly" versus "slow but gratis" genetic adaptations to *Plasmodium falciparum* malaria," *Human Molecular Genetics*, vol. 17, no. 6, pp. 789–799, 2008.

[53] D. Modiano, G. Luoni, B. S. Sirima et al., "Haemoglobin C protects against clinical *Plasmodium falciparum* malaria," *Nature*, vol. 414, no. 6861, pp. 305–308, 2001.

[54] R. Hutagalung, P. Wilairatana, S. Looareesuwan, G. M. Brittenham, M. Aikawa, and V. R. Gordeuk, "Influence of hemoglobin E trait on the severity of falciparum malaria," *Journal of Infectious Diseases*, vol. 179, no. 1, pp. 283–286, 1999.

[55] A. Ferreira, I. Marguti, I. Bechmann et al., "Sickle hemoglobin confers tolerance to plasmodium infection," *Cell*, vol. 145, no. 3, pp. 398–409, 2011.

[56] S. E. Francis, D. J. Sullivan, and D. E. Goldberg, "Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*," *Annual Review of Microbiology*, vol. 51, pp. 97–123, 1997.

[57] A. U. Orjih, H. S. Banyal, R. Chevli, and C. D. Fitch, "Hemin lyses malaria parasites," *Science*, vol. 214, no. 4521, pp. 667–669, 1981.

[58] A. Ferreira, J. Balla, V. Jeney, G. Balla, and M. P. Soares, "A central role for free heme in the pathogenesis of severe malaria: the missing link?" *Journal of Molecular Medicine*, vol. 86, no. 10, pp. 1097–1111, 2008.

[59] R. Gozzelino and M. P. Soares, "Heme sensitization to TNF-mediated programmed cell death," *Advances in Experimental Medicine and Biology*, vol. 691, pp. 211–219, 2011.

[60] M. Kristiansen, J. H. Graversen, C. Jacobsen et al., "Identification of the haemoglobin scavenger receptor," *Nature*, vol. 409, no. 6817, pp. 198–201, 2001.

[61] N. Maeda, F. Yang, and D. R. Barnett, "Duplication within the haptoglobin Hp2 gene," *Nature*, vol. 309, no. 5964, pp. 131–135, 1984.

[62] R. Asleh, J. Guetta, S. Kalet-Litman, R. Miller-Lotan, and A. P. Levy, "Haptoglobin genotype- and diabetes-dependent differences in iron-mediated oxidative stress in vitro and in vivo," *Circulation Research*, vol. 96, no. 4, pp. 435–441, 2005.

[63] M. Melamed-Frank, O. Lache, B. I. Enav et al., "Structure-function analysis of the antioxidant properties of haptoglobin," *Blood*, vol. 98, no. 13, pp. 3693–3698, 2001.

[64] S. Rogerson, "What is the relationship between haptoglobin, malaria, and anaemia?" *PLoS Medicine*, vol. 3, no. 5, article e200, 2006.

[65] V. R. R. Mendonça, N. F. Luza, N. J. G. Santosa et al., "Association between the haptoglobin and heme oxygenase 1 genetic profiles and soluble CD163 in susceptibility to and severity of human malaria," *Infection and Immunity*, vol. 80, no. 4, pp. 1445–1454, 2012.

[66] A. Pamplona, A. Ferreira, J. Balla et al., "Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria," *Nature Medicine*, vol. 13, no. 6, pp. 703–710, 2007.

[67] M. Exner, E. Minar, O. Wagner, and M. Schillinger, "The role of heme oxygenase-1 promoter polymorphisms in human disease," *Free Radical Biology and Medicine*, vol. 37, no. 8, pp. 1097–1104, 2004.

[68] T. Kimpara, A. Takeda, K. Watanabe et al., "Microsatellite polymorphism in the human heme oxygenase-1 gene promoter and its application in association studies with Alzheimer and Parkinson disease," *Human Genetics*, vol. 100, no. 1, pp. 145–147, 1997.

[69] Y.-H. Chen, S. J. Lin, M. W. Lin et al., "Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients," *Human Genetics*, vol. 111, no. 1, pp. 1–8, 2002.

[70] N. Yamada, M. Yamaya, S. Okinaga et al., "Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema," *American Journal of Human Genetics*, vol. 66, no. 1, pp. 187–195, 2000.

[71] C.-C. Sheu, R. Zhai, Z. Wang et al., "Heme Oxygenase-1 microsatellite polymorphism and haplotypes are associated with the development of acute respiratory distress syndrome," *Intensive Care Medicine*, vol. 35, no. 8, pp. 1343–1351, 2009.

[72] H. J. Chin, H. J. Cho, T. W. Lee et al., "The heme oxygenase-1 genotype is a risk factor to renal impairment of IgA nephropathy at diagnosis, which is a strong predictor of mortality," *Journal of Korean Medical Science*, vol. 24, supplement, pp. S30–37, 2009.

[73] F. A. D. T. G. Wagener, E. J. M. Toonen, L. Wigman et al., "HMOX1 promoter polymorphism modulates the relationship between disease activity and joint damage in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 58, no. 11, pp. 3388–3393, 2008.

[74] M. R. Sambo, M. J. Trovoada, C. Benchimol et al., "Transforming growth factor beta 2 and heme oxygenase 1 genes are risk factors for the cerebral malaria syndrome in Angolan children," *PLoS ONE*, vol. 5, no. 6, Article ID e11141, 2010.

[75] M. Takeda, M. Kikuchi, R. Ubalee et al., "Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to cerebral malaria in Myanmar," *Japanese Journal of Infectious Diseases*, vol. 58, no. 5, pp. 268–271, 2005.

[76] B. B. Andrade, T. Araújo-Santos, N. F. Luz et al., "Heme impairs prostaglandin E2 and TGF-β production by human mononuclear cells via Cu/Zn superoxide dismutase: insight into the pathogenesis of severe malaria," *Journal of Immunology*, vol. 185, no. 2, pp. 1196–1204, 2010.

[77] B. B. Andrade, A. Reis-Filho, S. M. Souza-Neto et al., "Plasma superoxide dismutase-1 as a surrogate marker of vivax malaria severity," *PLoS Neglected Tropical Diseases*, vol. 4, no. 4, articlee 650, 2010.

[78] M. Basu, A. K. Maji, A. Chakraborty et al., "Genetic association of Toll-like-receptor 4 and tumor necrosis factor-α polymorphisms with *Plasmodium falciparum* blood infection levels," *Infection, Genetics and Evolution*, vol. 10, no. 5, pp. 686–696, 2010.

[79] H. Kumar, T. Kawai, and S. Akira, "Toll-like receptors and innate immunity," *Biochemical and Biophysical Research Communications*, vol. 388, no. 4, pp. 621–625, 2009.

[80] C. A. Janeway and R. Medzhitov, "Innate immune recognition," *Annual Review of Immunology*, vol. 20, pp. 197–216, 2002.

[81] K. J. Ishii, S. Uematsu, and S. Akira, "'Toll' gates for future immunotherapy," *Current Pharmaceutical Design*, vol. 12, no. 32, pp. 4135–4142, 2006.

[82] M. B. B. McCall, M. G. Netea, C. C. Hermsen et al., "*Plasmodium falciparum* infection causes proinflammatory priming of human TLR responses," *Journal of Immunology*, vol. 179, no. 1, pp. 162–171, 2007.

[83] L. A. J. O'Neill and A. G. Bowie, "The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling," *Nature Reviews Immunology*, vol. 7, no. 5, pp. 353–364, 2007.

[84] F. M. S. Leoratti, L. Farias, F. P. Alves et al., "Variants in the toll-like receptor signaling pathway and clinical outcomes of malaria," *Journal of Infectious Diseases*, vol. 198, no. 5, pp. 772–780, 2008.

[85] G. Krishnegowda, A. M. Hajjar, J. Zhu et al., "Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum*: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity," *The Journal of Biological Chemistry*, vol. 280, no. 9, pp. 8606–8616, 2005.

[86] C. Coban, K. J. Ishii, T. Kawai et al., "Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin," *Journal of Experimental Medicine*, vol. 201, no. 1, pp. 19–25, 2005.

[87] P. Parroche, F. N. Lauw, N. Goutagny et al., "Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 6, pp. 1919–1924, 2007.

[88] N. W. J. Schröder and R. R. Schumann, "Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease," *Lancet Infectious Diseases*, vol. 5, no. 3, pp. 156–164, 2005.

[89] E. Noguchi, F. Nishimura, H. Fukai et al., "An association study of asthma and total serum immunoglobin E levels for Toll-like receptor polymorphisms in a Japanese population," *Clinical and Experimental Allergy*, vol. 34, no. 2, pp. 177–183, 2004.

[90] J.-J. Yim, H. W. Lee, H. S. Lee et al., "The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans," *Genes and Immunity*, vol. 7, no. 2, pp. 150–155, 2006.

[91] J. A. Greene, N. Sam-Agudu, C. C. John, R. O. Opoka, P. A. Zimmerman, and J. W. Kazura, "Toll-like receptor polymorphisms and cerebral malaria: TLR2 Δ22 polymorphism is associated with protection from cerebral malaria in a case control study," *Malaria Journal*, vol. 11, article 47, 2012.

[92] F. P. Mockenhaupt, J. P. Cramer, L. Hamann et al., "Toll-like receptor (TLR) polymorphisms in African children: common TLR-4 variants predispose to severe malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 1, pp. 177–182, 2006.

[93] F. P. Mockenhaupt, J. P. Cramer, L. Hamann et al., "Toll-Like Receptor (TLR) polymorphisms in African children:

common TLR-4 variants predispose to severe malaria," *Journal of Communicable Diseases*, vol. 38, no. 3, pp. 230–245, 2006.

[94] F. P. Mockenhaupt, L. Hamann, C. Von Gaertner et al., "Common polymorphisms of toll-like receptors 4 and 9 are associated with the clinical manifestation of malaria during pregnancy," *The Journal of Infectious Diseases*, vol. 194, no. 2, pp. 184–188, 2006.

[95] C. Coban, S. Uematsu, N. Arisue et al., "Pathological role of Toll-like receptor signaling in cerebral malaria," *International Immunology*, vol. 19, no. 1, pp. 67–79, 2007.

[96] B. S. Franklin, S. T. Ishizaka, M. Lamphier et al., "Therapeutical targeting of nucleic acid-sensing Toll-like receptors prevents experimental cerebral malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 9, pp. 3689–3694, 2011.

[97] S. Campino, J. Forton, S. Auburn et al., "TLR9 polymorphisms in African populations: no association with severe malaria, but evidence of cis-variants acting on gene expression," *Malaria Journal*, vol. 8, no. 1, article 44, 2009.

[98] S. Zakeri, S. Pirahmadi, A. A. Mehrizi, and N. D. Djadid, "Genetic variation of TLR-4, TLR-9 and TIRAP genes in Iranian malaria patients," *Malaria Journal*, vol. 10, article 77, 2011.

[99] A. H. Omar, M. Yasunami, A. Yamazaki et al., "Toll-like receptor 9 (TLR9) polymorphism associated with symptomatic malaria: a cohort study," *Malaria Journal*, vol. 11, no. 1, p. 168, 2012.

[100] M. Yamamoto, S. Sato, H. Hemmi et al., "Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4," *Nature*, vol. 420, no. 6913, pp. 324–329, 2002.

[101] C. C. Khor, F. O. Vannberg, S. J. Chapman et al., "Positive replication and linkage disequilibrium mapping of the chromosome 21q22.1 malaria susceptibility locus," *Genes and Immunity*, vol. 8, no. 7, pp. 570–576, 2007.

[102] N. O. Wilson, T. Bythwood, W. Solomon et al., "Elevated levels of IL-10 and G-CSF associated with asymptomatic malaria in pregnant women," *Infectious Diseases in Obstetrics and Gynecology*, vol. 2010, Article ID 317430, 7 pages, 2010.

[103] R. Medzhitov, D. S. Schneider, and M. P. Soares, "Disease tolerance as a defense strategy," *Science*, vol. 335, no. 6071, pp. 936–941, 2012.

[104] E. Seixas, R. Gozzelino, A. Chora et al., "Heme oxygenase-1 affords protection against noncerebral forms of severe malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 37, pp. 15837–15842, 2009.

[105] D. Kwiatkowski, "Genetic susceptibility to malaria getting complex," *Current Opinion in Genetics and Development*, vol. 10, no. 3, pp. 320–324, 2000.

[106] D. Kwiatkowski, A. V. S. Hill, I. Sambou et al., "TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria," *The Lancet*, vol. 336, no. 8725, pp. 1201–1204, 1990.

[107] N. D. Karunaweera, G. E. Grau, P. Gamage, R. Carter, and K. N. Mendis, "Dynamics of fever and serum levels of tumor necrosis factor are closely associated during clinical paroxysms in *Plasmodium vivax* malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 8, pp. 3200–3203, 1992.

[108] B. G. Mordmüller, W. G. Metzger, P. Juillard et al., "Tumor necrosis factor in *Plasmodium falciparum* malaria: high plasma level is associated with fever, but high production capacity is associated with rapid fever clearance," *European Cytokine Network*, vol. 8, no. 1, pp. 29–35, 1997.

[109] N. Depinay, J. F. Franetich, A. C. Grüner et al., "Inhibitory effect of TNF-α on malaria pre-erythrocytic stage development: influence of host hepatocyte/parasite combinations," *PLoS ONE*, vol. 6, no. 3, Article ID e17464, 2011.

[110] W. McGuire, A. V. S. Hill, C. E. M. Allsopp, B. M. Greenwood, and D. Kwjatkowski, "Variation in the TNF-α promoter region associated with susceptibility to cerebral malaria," *Nature*, vol. 371, no. 6497, pp. 508–511, 1994.

[111] T. G. Clark, M. Diakite, S. Auburn et al., "Tumor necrosis factor and lymphotoxin-α polymorphisms and severe malaria in African populations," *Journal of Infectious Diseases*, vol. 199, no. 4, pp. 569–575, 2009.

[112] C. G. Meyer, J. May, A. J. Luty, B. Lell, and P. G. Kremsner, "TNFα-308A associated with shorter intervals of *Plasmodium falciparum* reinfections," *Tissue Antigens*, vol. 59, no. 4, pp. 287–292, 2002.

[113] J. Wattavidanage, R. Carter, K. L. R. L. Perera et al., "TNFα*2 marks high risk of severe disease during *Plasmodium falciparum* malaria and other infections in Sri Lankans," *Clinical and Experimental Immunology*, vol. 115, no. 2, pp. 350–355, 1999.

[114] R. Ubalee, F. Suzuki, M. Kikuchi et al., "Strong association of a tumor necrosis factor-α promoter allele with cerebral malaria in Myanmar," *Tissue Antigens*, vol. 58, no. 6, pp. 407–410, 2001.

[115] H. A. Stirnadel, M. Stöckle, I. Felger, T. Smith, M. Tanner, and H. P. Beck, "Malaria infection and morbidity in infants in relation to genetic polymorphisms in Tanzania," *Tropical Medicine and International Health*, vol. 4, no. 3, pp. 187–193, 1999.

[116] L. M. Randall, E. Kenangalem, D. A. Lampah et al., "A study of the TNF/LTA/LTB locus and susceptibility to severe malaria in highland papuan children and adults," *Malaria Journal*, vol. 9, no. 1, article 302, 2010.

[117] S. Cabantous, O. Doumbo, S. Ranque et al., "Alleles 308A and 238A in the tumor necrosis factor alpha gene promoter do not increase the risk of severe malaria in children with *Plasmodium falciparum* infection in Mali," *Infection and Immunity*, vol. 74, no. 12, pp. 7040–7042, 2006.

[118] J. C. Knight, B. J. Keating, and D. P. Kwiatkowski, "Allele-specific repression of lymphotoxin-α by activated B cell factor-1," *Nature Genetics*, vol. 36, no. 4, pp. 394–399, 2004.

[119] M. Barbier, N. F. Delahaye, F. Fumoux, and P. Rihet, "Family-based association of a low producing lymphotoxin-α allele with reduced *Plasmodium falciparum* parasitemia," *Microbes and Infection*, vol. 10, no. 6, pp. 673–679, 2008.

[120] M. Diakite, T. G. Clark, S. Auburn et al., "A genetic association study in the Gambia using tagging polymorphisms in the major histocompatibility complex class III region implicates a HLA-B associated transcript 2 polymorphism in severe malaria susceptibility," *Human Genetics*, vol. 125, no. 1, pp. 105–109, 2009.

[121] G. Messer, U. Spengler, M. C. Jung et al., "Polymorphic structure of the tumor necrosis factor (TNF) locus: an NcoI polymorphism in the first intron of the human TNF-β gene correlates with a variant amino acid in position 26 and a reduced level of TNF-β production," *Journal of Experimental Medicine*, vol. 173, no. 1, pp. 209–219, 1991.

[122] B. A. Gyan, B. Goka, J. T. Cvetkovic et al., "Allelic polymorphisms in the repeat and promoter regions of the interleukin-4 gene and malaria severity in Ghanaian children," *Clinical*

and Experimental Immunology, vol. 138, no. 1, pp. 145–150, 2004.

[123] I. Naka, N. Nishida, J. Patarapotikul et al., "Identification of a haplotype block in the 5q31 cytokine gene cluster associated with the susceptibility to severe malaria," *Malaria Journal*, vol. 8, no. 1, article 232, 2009.

[124] P. Rihet, Y. Traoré, L. Abel, C. Aucan, T. Traoré-Leroux, and F. Fumoux, "Malaria in humans: *Plasmodium falciparum* blood infection levels are linked to chromosome 5q31-q33," *American Journal of Human Genetics*, vol. 63, no. 2, pp. 498–505, 1998.

[125] A. Garcia, S. Marquet, B. Bucheton et al., "Linkage analysis of blood *Plasmodium falciparum* levels: interest of the 5q31-q33 chromosome region," *American Journal of Tropical Medicine and Hygiene*, vol. 58, no. 6, pp. 705–709, 1998.

[126] A. Sakuntabhai, R. Ndiaye, I. Casadémont et al., "Genetic determination and linkage mapping of *Plasmodium falciparum* malaria related traits in Senegal," *PLoS ONE*, vol. 3, no. 4, Article ID e2000, 2008.

[127] J. Milet, G. Nuel, L. Watier et al., "Genome wide linkage study, using a 250K SNP map, of *Plasmodium falciparum* infection and mild malaria attack in a senegalese population," *PLoS ONE*, vol. 5, no. 7, Article ID e11616, 2010.

[128] F. Y. Zeyrek, M. A. Kurcer, D. Zeyrek, and Z. Simsek, "Parasite density and serum cytokine levels in *Plasmodium vivax* malaria in Turkey," *Parasite Immunology*, vol. 28, no. 5, pp. 201–207, 2006.

[129] S. Cabantous, B. Poudiougou, A. A. Oumar et al., "Genetic evidence for the aggravation of *Plasmodium falciparum* malaria by interleukin 4," *Journal of Infectious Diseases*, vol. 200, no. 10, pp. 1530–1539, 2009.

[130] D. Torre, F. Speranza, M. Giola, A. Matteelli, R. Tambini, and G. Biondi, "Role of Th1 and Th2 cytokines in immune response to uncomplicated *Plasmodium falciparum* malaria," *Clinical and Diagnostic Laboratory Immunology*, vol. 9, no. 2, pp. 348–351, 2002.

[131] V. Pravica, A. Asderakis, C. Perrey, A. Hajeer, P. J. Sinnott, and I. V. Hutchinson, "In vitro production of IFN-γ correlates with CA repeat polymorphism in the human IFN-γ gene," *European Journal of Immunogenetics*, vol. 26, no. 1, pp. 1–3, 1999.

[132] V. Pravica, C. Perrey, A. Stevens, J. H. Lee, and I. V. Hutchinson, "A single nucleotide polymorphism in the first intron of the human IFN-γ gene: absolute correlation with a polymorphic CA microsatellite marker of high IFN-γ production," *Human Immunology*, vol. 61, no. 9, pp. 863–866, 2000.

[133] O. Kock, K. Rockett, M. Jallow, M. Pinder, F. Sisay-Joof, and D. Kwiatkowski, "Investigation of malaria susceptibility determinants in the IFNG/IL26/IL22 genomic region," *Genes and Immunity*, vol. 6, no. 4, pp. 312–318, 2005.

[134] S. Cabantous, B. Poudiougou, A. Traore et al., "Evidence that interferon-γ plays a protective role during cerebral malaria," *Journal of Infectious Diseases*, vol. 192, no. 5, pp. 854–860, 2005.

[135] J. Ohashi, I. Naka, J. Patarapotikul, H. Hananantachai, S. Looareesuwan, and K. Tokunaga, "A single-nucleotide substitution from C to T at position-1055 in the IL-13 promoter is associated with protection from severe malaria in Thailand," *Genes and Immunity*, vol. 4, no. 7, pp. 528–531, 2003.

[136] R. L. Dewasurendra, P. Suriyaphol, and S. D. Fernando, "Genetic polymorphisms associated with anti-malarial antibody levels in a low and unstable malaria transmission area in southern Sri Lanka," *Malaria Journal*, vol. 11, article 281, 2012.

[137] C. Phawong, C. Ouma, P. Tangteerawatana et al., "Haplotypes of IL12B promoter polymorphisms condition susceptibility to severe malaria and functional changes in cytokine levels in Thai adults," *Immunogenetics*, vol. 62, no. 6, pp. 345–356, 2010.

[138] L. Zhang, D. Prather, J. V. Eng et al., "Polymorphisms in genes of interleukin 12 and its receptors and their association with protection against severe malarial anaemia in children in western Kenya," *Malaria Journal*, vol. 9, no. 1, article 87, 2010.

[139] I. Naka, J. Patarapotikul, K. Tokunaga, H. Hananantachai, N. Tsuchiya, and J. Ohashi, "A replication study of the association between the IL12B promoter allele CTCTAA and susceptibility to cerebral malaria in Thai population," *Malaria Journal*, vol. 8, no. 1, article 290, 2009.

[140] S. Marquet, O. Doumbo, S. Cabantous et al., "A functional promoter variant in IL12B predisposes to cerebral malaria," *Human Molecular Genetics*, vol. 17, no. 14, pp. 2190–2195, 2008.

[141] G. Morahan, C. S. Boutlis, D. Huang et al., "A promoter polymorphism in the gene encoding interleukin-12 p40 (IL12B) is associated with mortality from cerebral malaria and with reduced nitric oxide production," *Genes and Immunity*, vol. 3, no. 7, pp. 414–418, 2002.

[142] D. G. Marsh, J. D. Neely, D. R. Breazeale et al., "Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations," *Science*, vol. 264, no. 5162, pp. 1152–1156, 1994.

[143] S. Romagnani, "Biology of human T(H)1 and T(H)2 cells," *Journal of Clinical Immunology*, vol. 15, no. 3, pp. 121–129, 1995.

[144] E. E. Nakayama, Y. Hoshino, X. Xin et al., "Polymorphism in the interleukin-4 promoter affects acquisition of human immunodeficiency virus type 1 syncytium-inducing phenotype," *Journal of Virology*, vol. 74, no. 12, pp. 5452–5459, 2000.

[145] D. G. Marsh, J. D. Neely, D. R. Breazeale et al., "Total serum IgE levels and chromosome 5q," *Clinical and Experimental Allergy*, vol. 25, supplement 2, pp. 79–83, 1995.

[146] I. Suzuki, N. Hizawa, E. Yamaguchi, and Y. Kawakami, "Association between a C+33T polymorphism in the IL-4 promoter region and total serum IgE levels," *Clinical and Experimental Allergy*, vol. 30, no. 12, pp. 1746–1749, 2000.

[147] F. Verra, P. Avellino, G. Bancone, V. Mangano, and D. Modiano, "Genetic epidemiology of susceptibility to malaria: not only academic exercises," *Parassitologia*, vol. 50, no. 1-2, pp. 147–150, 2008.

[148] A. J. Walley, C. Aucan, D. Kwiatkowski, and A. V. S. Hill, "Interleukin-1 gene cluster polymorphins and susceptibility to clinical malaria in a Gambian case-control study," *European Journal of Human Genetics*, vol. 12, no. 2, pp. 132–138, 2004.

[149] C. Aucan, A. J. Walley, B. J. W. Hennig et al., "Interferon-alpha receptor-1 (IFNAR1) variants are associated with protection against cerebral malaria in The Gambia," *Genes and Immunity*, vol. 4, no. 4, pp. 275–282, 2003.

[150] C. Ouma, G. C. Davenport, G. A. Awandare et al., "Polymorphic variability in the interleukin (IL)-1β promoter conditions susceptibility to severe malarial anemia and functional changes in IL-1β production," *Journal of Infectious Diseases*, vol. 198, no. 8, pp. 1219–1226, 2008.

[151] J. Rogus, J. D. Beck, S. Offenbacher et al., "IL1B gene promoter haplotype pairs predict clinical levels of interleukin-1β and C-reactive protein," *Human Genetics*, vol. 123, no. 4, pp. 387–398, 2008.

[152] I. Lalani, K. Bhol, and A. R. Ahmed, "Interleukin-10: biology, role in inflammation and autoimmunity," *Annals of Allergy*, vol. 79, no. 6, pp. 469–483, 1997.

[153] J. Eskdale and G. Gallagher, "A polymorphic dinucleotide repeat in the human IL-10 promoter," *Immunogenetics*, vol. 42, no. 5, pp. 444–445, 1995.

[154] C. Ouma, G. C. Davenport, T. Were et al., "Haplotypes of IL-10 promoter variants are associated with susceptibility to severe malarial anemia and functional changes in IL-10 production," *Human Genetics*, vol. 124, no. 5, pp. 515–524, 2008.

[155] T. S. Medina et al., "Increased interleukin-10 and interferon-â levels in *Plasmodium vivax* malaria suggest a reciprocal regulation which is not altered by IL-10 gene promoter polymorphism," *Malaria Journal*, vol. 10, article 264, 2011.

[156] J. N. Wilson, K. Rockett, M. Jallow et al., "Analysis of IL10 haplotypic associations with severe malaria," *Genes and Immunity*, vol. 6, no. 6, pp. 462–466, 2005.

[157] K. Omi, J. Ohashi, J. Patarapotikul et al., "Fcγ receptor IIA and IIIB polymorphisms are associated with susceptibility to cerebral malaria," *Parasitology International*, vol. 51, no. 4, pp. 361–366, 2002.

[158] Y. P. Shi, B. L. Nahlen, S. Kariuki et al., "Fc-γ receptor IIa (CD32) polymorphism is associated with protection of infants against high-density *Plasmodium falciparum* infection. VII. Asembo bay cohort project," *Journal of Infectious Diseases*, vol. 184, no. 1, pp. 107–111, 2001.

[159] E. M. Braga, K. K. G. Scopel, N. T. Komatsu, M. da Silva-Nunes, and M. U. Ferreira, "Polymorphism of the Fcgamma receptor IIA and malaria morbidity," *Journal of Molecular and Genetic Medicine*, vol. 1, no. 1, pp. 5–10, 2005.

[160] J. E. Salmon, J. C. Edberg, and R. P. Kimberly, "Fcγ receptor III on human neutrophils. Allelic variants have functionally distinct capacities," *Journal of Clinical Investigation*, vol. 85, no. 4, pp. 1287–1295, 1990.

[161] L. A. M. Sanders, R. G. Feldman, M. M. Voorhorst-Ogink et al., "Human immunoglobulin G (IgG) Fc receptor IIA (CD32) polymorphism and IgG2- mediated bacterial phagocytosis by neutrophils," *Infection and Immunity*, vol. 63, no. 1, pp. 73–81, 1995.

[162] C. Aucan, Y. Traoré, F. Tall et al., "High immunoglobulin G2 (IgG2) and low IgG4 levels are associated with human resistance to *Plasmodium falciparum* malaria," *Infection and Immunity*, vol. 68, no. 3, pp. 1252–1258, 2000.

[163] C. Ouma, C. C. Keller, D. A. Opondo et al., "Association of FCγ receptor IIA (CD32) polymorphism with malarial anemia and high-density parasitemia in infants and young children," *American Journal of Tropical Medicine and Hygiene*, vol. 74, no. 4, pp. 573–577, 2006.

[164] G. S. Cooke, C. Aucan, A. J. Walley et al., "Association of Fcγ receptor IIa (CD32) polymorphism with severe malaria in West Africa," *American Journal of Tropical Medicine and Hygiene*, vol. 69, no. 6, pp. 565–568, 2003.

[165] A. Nasr, N. C. Iriemenam, M. Troye-Blomberg et al., "Fc gamma receptor IIa (CD32) polymorphism and antibody responses to asexual blood-stage antigens of *Plasmodium falciparum* malaria in Sudanese patients," *Scandinavian Journal of Immunology*, vol. 66, no. 1, pp. 87–96, 2007.

[166] M. C. Levesque, M. R. Hobbs, C. W. O'Loughlin et al., "Malaria severity and human nitric oxide synthase type 2 (NOS2) promoter haplotypes," *Human Genetics*, vol. 127, no. 2, pp. 163–182, 2010.

[167] P. Balmer, H. M. Phillips, A. E. Maestre, F. A. McMonagle, and R. S. Phillips, "The effect of nitric oxide on the growth of *Plasmodium falciparum*, P. chabaudi and P. berghei in vitro," *Parasite Immunology*, vol. 22, no. 2, pp. 97–106, 2000.

[168] S. Serirom, W. H. Raharjo, K. Chotivanich, S. Loareesuwan, P. Kubes, and M. Ho, "Anti-adhesive effect of nitric oxide on *Plasmodium falciparum* cytoadherence under flow," *American Journal of Pathology*, vol. 162, no. 5, pp. 1651–1660, 2003.

[169] J. F. J. Kun, B. Mordmüller, B. Lell, L. G. Lehman, D. Luckner, and P. G. Kremsner, "Polymorphism in promoter region of inducible nitric oxide synthase gene and protection against malaria," *The Lancet*, vol. 351, no. 9098, pp. 265–266, 1998.

[170] D. Burgner, W. Xu, K. Rockett et al., "Inducible nitric oxide synthase polymorphism and fatal cerebral malaria," *The Lancet*, vol. 352, no. 9135, pp. 1193–1194, 1998.

[171] M. C. Levesque, M. Hobbs, and N. Anstey, "Nitric oxide synthase type 2 promoter polymorphisms, nitric oxide production, and disease severity in Tanzanian children with malaria," *Journal of Infectious Diseases*, vol. 181, no. 6, pp. 1994–2002, 2000.

[172] M. R. Hobbs, V. Udhayakumar, M. C. Levesque et al., "A new NOS2 promoter polymorphism associated with increased nitric oxide production and protection from severe malaria in Tanzanian and Kenyan children," *The Lancet*, vol. 360, no. 9344, pp. 1468–1475, 2002.

[173] L. H. Miller, M. F. Good, and G. Milon, "Malaria pathogenesis," *Science*, vol. 264, no. 5167, pp. 1878–1883, 1994.

[174] J. K. Patnaik, B. S. Das, S. K. Mishra, S. Mohanty, S. K. Satpathy, and D. Mohanty, "Vascular clogging, mononuclear cell margination, and enhanced vascular permeability in the pathogenesis of human cerebral malaria," *The American Journal of Tropical Medicine and Hygiene*, vol. 51, no. 5, pp. 642–647, 1994.

[175] B. C. Urban, D. J. P. Ferguson, A. Pain et al., "*Plasmodium falciparum*infected erythrocytes modulate the maturation of dendritic cells," *Nature*, vol. 400, no. 6739, pp. 73–77, 1999.

[176] C. F. Ockenhouse, "The molecular basis for the cytoadherence of *Plasmodium falciparum*-infected erythrocytes to endothelium," *Seminars in Cell and Developmental Biology*, vol. 4, no. 5, pp. 297–303, 1993.

[177] C. F. Ockenhouse, R. Betageri, T. A. Springer, and D. E. Staunton, "*Plasmodium falciparum*-infected erythrocytes bind ICAM-1 at a site distinct from LFA-1, Mac-1, and human rhinovirus," *Cell*, vol. 68, no. 1, pp. 63–69, 1992.

[178] C. F. Ockenhouse, T. Tegoshi, Y. Maeno et al., "Human vascular endothelial cell adhesion receptors for *Plasmodium falciparum*-infected erythrocytes: roles for endothelial leukocyte adhesion molecule 1 and vascular cell adhesion molecule 1," *Journal of Experimental Medicine*, vol. 176, no. 4, pp. 1183–1189, 1992.

[179] I. W. Sherman, S. Eda, and E. Winograd, "Cytoadherence and sequestration in *Plasmodium falciparum*: defining the ties that bind," *Microbes and Infection*, vol. 5, no. 10, pp. 897–909, 2003.

[180] J. A. Rowe, J. M. Moulds, C. I. Newbold, and L. H. Miller, "*P. falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1," *Nature*, vol. 388, no. 6639, pp. 292–295, 1997.

[181] G. B. Nash, B. M. Cooke, J. Carlson, and M. Wahlgren, "Rheological properties of rosettes formed by red blood cells parasitized by *Plasmodium falciparum*," *British Journal of Haematology*, vol. 82, no. 4, pp. 757–763, 1992.

[182] J. A. Rowe, J. Obiero, K. Marsh, and A. Raza, "Short report: positive correlation between rosetting and parasitemia in *Plasmodium falciparum* clinical isolates," *American Journal of Tropical Medicine and Hygiene*, vol. 66, no. 5, pp. 458–460, 2002.

[183] A.-M. Deans and J. A. Rowe, "*Plasmodium falciparum*: rosettes do not protect merozoites from invasion-inhibitory antibodies," *Experimental Parasitology*, vol. 112, no. 4, pp. 269–273, 2006.

[184] R. Rout, G. Dhangadamajhi, B. N. Mohapatra, S. K. Kar, and M. Ranjit, "High CR1 level and related polymorphic variants are associated with cerebral malaria in eastern-India," *Infection, Genetics and Evolution*, vol. 11, no. 1, pp. 139–144, 2011.

[185] G. D. H. Turner, H. Morrison, M. Jones et al., "An immunohistochemical study of the pathology of fatal malaria: evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration," *American Journal of Pathology*, vol. 145, no. 5, pp. 1057–1069, 1994.

[186] C. Newbold, P. Warn, G. Black et al., "Receptor-specific adhesion and clinical disease in *Plasmodium falciparum*," *American Journal of Tropical Medicine and Hygiene*, vol. 57, no. 4, pp. 389–398, 1997.

[187] A. E. Fry, S. Auburn, M. Diakite et al., "Variation in the ICAM1 gene is not associated with severe malaria phenotypes," *Genes and Immunity*, vol. 9, no. 5, pp. 462–469, 2008.

[188] S. D. Marlin and T. A. Springer, "Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1)," *Cell*, vol. 51, no. 5, pp. 813–819, 1987.

[189] M. Baratin, S. Roetynck, B. Pouvelle et al., "Dissection of the role of PfEMP1 and ICAM-1 in the sensing of *Plasmodium falciparum*-infected erythrocytes by natural killer cells," *PLoS ONE*, vol. 2, no. 2, article e228, 2007.

[190] D. Fernandez-Reyes, A. G. Craig, S. A. Kyes et al., "A high frequency African coding polymorphism in the N-terminal domain of ICAM-1 predisposing to cerebral malaria in Kenya," *Human Molecular Genetics*, vol. 6, no. 8, pp. 1357–1360, 1997.

[191] J. F. Kun, J. Klabunde, B. Lell et al., "Association of the ICAM:1(KILIFI) mutation with protection against severe malaria in lambarene, gabon," *The American Journal of Tropical Medicine and Hygiene*, vol. 61, no. 5, pp. 776–779, 1999.

[192] O. K. Amodu, R. A. Gbadegesin, S. A. Ralph et al., "*Plasmodium falciparum* malaria in south-west Nigerian children: is the polymorphism of ICAM-1 and E-selectin genes contributing to the clinical severity of malaria?" *Acta Tropica*, vol. 95, no. 3, pp. 248–255, 2005.

[193] R. Bellamy, D. Kwiatkowski, and A. V. S. Hill, "Absence of an association between intercellular adhesion molecule 1, complement receptor 1 and interleukin 1 receptor antagonist gene polymorphisms and severe malaria in a West African population," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 92, no. 3, pp. 312–316, 1998.

[194] J. Ohashi, I. Naka, J. Patarapotikul, H. Hananantachai, S. Looareesuwan, and K. Tokunaga, "Absence of association between the allele coding methionine at position 29 in the N-terminal domain of ICAM-1 (ICAM-1Kilifi) and severe malaria in the northwest of Thailand," *Japanese Journal of Infectious Diseases*, vol. 54, no. 3, pp. 114–116, 2001.

[195] R. Ndiaye, A. Sakuntabhai, I. Casadémont et al., "Genetic study of ICAM1 in clinical malaria in Senegal," *Tissue Antigens*, vol. 65, no. 5, pp. 474–480, 2005.

[196] G. Ayodo, A. L. Price, A. Keinan et al., "Combining evidence of natural selection with association analysis increases power to detect malaria-resistance variants," *American Journal of Human Genetics*, vol. 81, no. 2, pp. 234–242, 2007.

[197] N. E. Jenkins, T. W. Mwangi, M. Kortok, K. Marsh, A. G. Craig, and T. H. Williams, "A polymorphism of intercellular adhesion molecule-1 is associated with a reduced incidence of nonmalarial febrile illness in Kenyan children," *Clinical Infectious Diseases*, vol. 41, no. 12, pp. 1817–1819, 2005.

[198] J. Chilongola, S. Balthazary, M. Mpina, M. Mhando, and E. Mbugi, "CD36 deficiency protects against malarial anaemia in children by reducing *Plasmodium falciparum*-infected red blood cell adherence to vascular endothelium," *Tropical Medicine and International Health*, vol. 14, no. 7, pp. 810–816, 2009.

[199] D. Kajeguka, C. Mwanziva, M. Daou et al., "CD36 c.1264 T > G null mutation impairs acquisition of IgG antibodies to *Plasmodium falciparum* $MSP1_{19}$ antigen and is associated with higher malaria incidences in Tanzanian children," *Scandinavian Journal of Immunology*, vol. 75, no. 3, pp. 355–360, 2012.

[200] T. J. Aitman, L. D. Cooper, P. J. Norsworthy et al., "Malaria susceptibility and CD36 mutation," *Nature*, vol. 405, no. 6790, pp. 1015–1016, 2000.

[201] A. Pain, B. C. Urban, O. Kai et al., "A non-sense mutation in Cd36 gene is associated with protection from severe malaria," *The Lancet*, vol. 357, no. 9267, pp. 1502–1503, 2001.

[202] S. Sinha, T. Qidwai, K. Kanchan et al., "Variations in host genes encoding adhesion molecules and susceptibility to falciparum malaria in India," *Malaria Journal*, vol. 7, article 250, 2008.

[203] K. Omi, J. Ohashi, I. Naka et al., "Polymorphisms of CD36 in Thai malaria patients," *The Southeast Asian journal of tropical medicine and public health*, vol. 33, supplement 3, pp. 1–4, 2002.

[204] C. J. Treutiger, A. Heddini, V. Fernandez, W. A. Muller, and M. Wahlgren, "PECAM-1/CD31, an endothelial receptor for binding *Plasmodium falciparum*- infected erythrocytes," *Nature Medicine*, vol. 3, no. 12, pp. 1405–1408, 1997.

[205] C. Casals-Pascual, S. Allen, A. Allen et al., "Short report: codon 125 polymorphism of CD31 and susceptibility to malaria," *American Journal of Tropical Medicine and Hygiene*, vol. 65, no. 6, pp. 736–737, 2001.

[206] M. Kikuchi, S. Looareesuwan, R. Ubalee et al., "Association of adhesion molecule PECAM-1/CD31 polymorphism with susceptibility to cerebral malaria in Thais," *Parasitology International*, vol. 50, no. 4, pp. 235–239, 2001.

[207] J. P. Newton, C. D. Buckley, E. Y. Jones, and D. L. Simmons, "Residues on both faces of the first immunoglobulin fold contribute to homophilic binding sites of PECAM-1/CD31," *Journal of Biological Chemistry*, vol. 272, no. 33, pp. 20555–20563, 1997.

[208] F. Liao, J. Ali, T. Greene, and W. A. Muller, "Soluble domain 1 of platelet-endothelial cell adhesion molecule (PECAM) is sufficient to block transendothelial migration in vitro and in vivo," *The Journal of Experimental Medicine*, vol. 185, no. 7, pp. 1349–1357, 1997.

[209] M. Krych-Goldberg, J. M. Moulds, and J. P. Atkinson, "Human complement receptor type 1 (CR1) binds to a major malarial adhesin," *Trends in Molecular Medicine*, vol. 8, no. 11, pp. 531–537, 2002.

[210] M. Krych-Goldberg and J. P. Atkinson, "Structure-function relationships of complement receptor type 1," *Immunological Reviews*, vol. 180, pp. 112–122, 2001.

[211] J. A. Rowe, S. J. Rogerson, A. Raza et al., "Mapping of the region of complement receptor (CR) 1 required for *Plasmodium falciparum* rosetting and demonstration of the importance of Cr1 in rosetting in field isolates," *Journal of Immunology*, vol. 165, no. 11, pp. 6341–6346, 2000.

[212] J. A. Stoute, "Complement-regulatory proteins in severe malaria: too little or too much of a good thing?" *Trends in Parasitology*, vol. 21, no. 5, pp. 218–223, 2005.

[213] A. Heddini, F. Pettersson, O. Kai et al., "Fresh isolates from children with severe *Plasmodium falciparum* malaria bind to multiple receptors," *Infection and Immunity*, vol. 69, no. 9, pp. 5849–5856, 2001.

[214] D. J. Birmingham and L. A. Hebert, "CR1 and CR1-like: the primate immune adherence receptors," *Immunological Reviews*, vol. 180, pp. 100–111, 2001.

[215] J. A. Rowe, A. Raza, D. A. Diallo et al., "Erythrocyte CR1 expression level does not correlate with a HindIII restriction fragment length polymorphism in Africans; implications for studies on malaria susceptibility," *Genes and Immunity*, vol. 3, no. 8, pp. 497–500, 2002.

[216] E. Nagayasu, M. Ito, M. Akaki et al., "CR1 density polymorphism on erythrocytes of falciparum malaria patients in Thailand," *American Journal of Tropical Medicine and Hygiene*, vol. 64, no. 1-2, pp. 1–5, 2001.

[217] I. A. Cockburn, M. J. Mackinnon, A. O'Donnell et al., "A human complement receptor 1 polymorphism that reduces *Plasmodium falciparum* rosetting confers protection against severe malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 1, pp. 272–277, 2004.

[218] P. Teeranaipong, J. Ohashi, J. Patarapotikul et al., "A functional single-nucleotide polymorphism in the CR1 promoter region contributes to protection against cerebral malaria," *Journal of Infectious Diseases*, vol. 198, no. 12, pp. 1880–1891, 2008.

[219] D. M. Tobin, F. J. Roca, S. F. Oh et al., "Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections," *Cell*, vol. 148, no. 3, pp. 434–446, 2012.

5.2 MANUSCRITO II

Association between the *Haptoglobin* and *Heme Oxygenase-1* Genetic Profiles and Soluble CD163 in Susceptibility to and Severity of Human Malaria.

Este trabalho investiga associações entre alterações genéticas e biomarcadores plasmáticos na via de metabolização do heme com os desfechos clínicos na malária por *P. vivax*.

Resumo dos resultados: Repetições (GT)n longas do polimorfismo microssatélite da *HMOX1*, o alelo *Hp2* e o genótipo *Hp2.2* da haptoglobina estão associados com o grupo de malária vivax sintomática quando comparado com malária assintomática e controles. Além disso, níveis plasmáticos elevados de heme, haptoglobina, heme oxigenase-1 e sCD163 estão associados com uma susceptibilidade maior para malária aguda.

Este trabalho foi publicado no periódico internacional *Infection and Immunity.*

Association between the Haptoglobin and Heme Oxygenase 1 Genetic Profiles and Soluble CD163 in Susceptibility to and Severity of Human Malaria

Vitor R. R. Mendonça,[a,b] Nívea F. Luz,[a,b] Nadja J. G. Santos,[a] Valéria M. Borges,[a,b,c] Marilda S. Gonçalves,[a,d] Bruno B. Andrade,[e] and Manoel Barral-Netto[a,b,c]

Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Bahia, Brazil[a]; Faculdade de Medicina, Universidade Federal da Bahia, Salvador, Bahia, Brazil[b]; Instituto Nacional de Ciência e Tecnologia de Investigação em Imunologia (iii-INCT), São Paulo, Brazil[c]; Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, Bahia, Brazil[d]; and Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA[e]

Intravascular hemolysis is a hallmark event in the immunopathology of malaria that results in increased systemic concentrations of free hemoglobin (Hb). The oxidation of Hb by free radicals causes the release of heme, which amplifies inflammation. To circumvent the detrimental effects of free heme, hosts have developed several homeostatic mechanisms, including the enzyme haptoglobin (Hp), which scavenges cell-free Hb, the monocyte receptor CD163, which binds to Hb-Hp complexes, and heme oxygenase-1 (HO-1), which degrades intracellular free heme. We tested the association between these three main components of the host response to hemolysis and susceptibility to malaria in a Brazilian population. The genetic profiles of the *HMOX1* and *Hp* genes and the plasma levels of a serum inflammatory marker, the soluble form of the CD163 receptor (sCD163), were studied in 264 subjects, including 78 individuals with symptomatic malaria, 106 individuals with asymptomatic malaria, and 80 uninfected individuals. We found that long (GT)*n* repeats in the microsatellite polymorphism region of the *HMOX1* gene, the *Hp2* allele, and the *Hp2.2* genotype were associated with symptomatic malaria. Moreover, increased plasma concentrations of heme, Hp, HO-1, and sCD163 were associated with susceptibility to malaria. The validation of these results could support the development of targeted therapies and aid in reducing the severity of malaria.

Malaria infection has high morbidity and mortality rates worldwide. During the blood stage of malarial infection, hemoglobin (Hb) is released from red blood cells that have ruptured due to *Plasmodium* multiplication (27). This unique characteristic of the *Plasmodium* life cycle leads to increased concentrations of cell-free Hb in the circulation because of intravascular hemolysis and the possible release of the heme prosthetic group from hemoglobin (25). Free heme is highly harmful to cells and tissues, as it can induce oxidative stress, cytotoxicity and inflammation (25), and cell death (30). Patients with severe malaria may exhibit high circulating levels of free heme, which impairs regulatory responses and can cause inflammatory imbalances (1). Under homeostatic conditions, haptoglobin (Hp) can rapidly scavenge cell-free Hb by forming the stable Hb-Hp complex, which is recognized and internalized by the CD163 receptor expressed by monocytes and macrophages in the red pulp of the spleen. Once internalized, the heme is usually degraded by the antioxidant enzyme heme oxygenase-1 (HO-1) (39). A thorough understanding of the factors and pathways that control the accumulation of free heme and the determinants of the unfavorable events that are triggered by this molecule can drive the development of novel therapeutic approaches to treat malaria and other hemolytic diseases.

Haptoglobin is a tetrameric protein ($\alpha 2\beta 2$) that is characterized by α-chain heterogeneity due to an intragenic duplication that resulted in two different alleles, *Hp2* and *Hp1* (including two subvariants, *Hp1F* and *Hp1S*). The diversity in the *Hp* phenotypes causes different binding affinities for cell-free Hb (*Hp1.1* > *Hp1.2* > *Hp2.2*) and CD163 (*Hp2.2* > *Hp1.2* > *Hp1.1*) (39). Additionally, polymorphisms in the *Hp* gene have been associated with different functional capabilities and organic responses, including alterations in immune regulation, oxidative stress, and iron delocalization within monocytes (8, 9, 31, 42–44, 54). Thus, it is necessary to consider the strategies used to study the mechanisms associated with heme regulation by HO-1, Hp, and the Hp receptor, CD163, and their contribution to the susceptibility to malaria.

The haptoglobin receptor CD163 is a member of a group of B cysteine-rich scavenger membrane receptors that is expressed on monocytes and macrophages and has been linked to inflammation. The soluble form of the CD163 receptor (sCD163) is a surrogate for its cellular expression, and sCD163 levels are elevated in many inflammatory processes (18, 29, 33, 45, 46, 50–52, 60, 66). Only one study has shown that sCD163 levels are more elevated in uncomplicated falciparum malaria than in severe malarial anemia and cerebral malaria, and all malaria patients have higher levels of sCD163 than uninfected individuals (41).

In experimental models of malaria, the induction of HO-1 is mostly associated with increased tolerance to *Plasmodium* infection (26, 53) as a result of the ability of HO-1 to control nonspe-

Received 13 September 2011 **Returned for modification** 29 October 2011
Accepted 7 January 2012
Published ahead of print 30 January 2012
Editor: J. H. Adams
Address correspondence to Manoel Barral-Netto, mbarral@bahia.fiocruz.br.
B.B.A. and M.B.-N. are senior authors and contributed equally to the article.
Copyright © 2012, American Society for Microbiology. All Rights Reserved.
doi:10.1128/IAI.05933-11

cific tissue damage and immunopathology by reducing inflammation. However, a few studies have linked *HMOX1* (*Homo sapiens*; P09601) gene polymorphisms to malaria susceptibility in humans (40, 59, 63). Notably, a (GT)*n* dinucleotide length polymorphism has been associated with varied expression of HO-1 (24). It has been suggested that there is a higher expression of HO-1 mRNA in patients with short (GT)*n* repeats in the *HMOX1* gene than in patients with long (GT)*n* repeats (24). Although a recent study showed an association between short (GT)*n* dinucleotide length and cerebral malaria (63), other investigations have not found any correlation between (GT)*n* dinucleotide length and malaria susceptibility (40). Therefore, there is no definitive evidence that links the length of the (GT)*n* dinucleotide repeats in the *HMOX1* gene with the severity of human malaria.

The main goal of this study was to investigate the Hp, HO-1, and sCD163 pathways that are involved in heme metabolism during malaria-induced intravascular hemolysis by analyzing genotypes and protein plasma levels in patients from the Brazilian Amazon. We tested whether different *Hp* genotypes and *HMOX1* microsatellite polymorphisms are associated with susceptibility to malaria. We have demonstrated that individuals presenting with symptomatic malaria more often have the *Hp2.2* genotype, which has previously been associated with a lower Hp binding affinity for cell-free Hb (39). In addition, we show that long (GT)*n* dinucleotide repeats in the *HMOX1* gene are associated with decreased concentrations of plasma HO-1, elevated disease susceptibility, and increased plasma levels of Hp and sCD163 in malaria patients. Furthermore, we address the association between sCD163 and malaria symptomatology. Thus, our findings expand to humans the current concept from experimental models that the susceptibility to malaria is indeed closely linked to specific determinants involved in heme metabolism.

MATERIALS AND METHODS

Ethics. This study is part of a project that was previously approved by the Ethical Committee of the São Lucas University, Rondônia, Brazil. All of the participants or their legal guardians provided informed consent before entering the study. The clinical investigations were conducted in accordance with the principles expressed in the 1975 Declaration of Helsinki, as revised in 2000.

Subjects. This study was a retrospective analysis of 264 subjects from the localities of Demarcação (8°10′04.12″S, 62°46′52.33″W) and Buritis (10°12′43″S, 63°49′44″W) in Rondônia State in the Brazilian Amazon. The subjects were studied between 2006 and 2007. The study sample includes 78 subjects with symptomatic malaria, 106 subjects with asymptomatic malaria, and 80 uninfected individuals. These individuals have already been analyzed by our group in other studies (1, 3–7). Active and passive case detections were performed using both microscopy and nested PCR. The symptomatic individuals promptly received antimalarial treatment, and those with asymptomatic infection at the time of enrollment in the study were followed for up to 30 days and subsequently classified as suffering from either symptomatic or asymptomatic malaria.

Genetic experiments. DNA was extracted from 200 μl of peripheral blood using a standard Qiagen DNA blood minikit (Valencia, CA) according to the manufacturer's protocol. The *Hp* genotypes were determined by allele-specific PCR as described by Yano et al. (70). The identification of the *Hp1F*, *Hp1S*, and *Hp2* alleles was based on the analysis of products from three independent PCRs. The PCR products were analyzed by electrophoresing them on 1% agarose gels under nondenaturing conditions. The products were then detected by staining with ethidium bromide and visualized under a UV light.

The 5′-flanking region of the *HMOX1* gene, which contains (GT)*n* repeats, was amplified with the forward primer 5′-AGAGCCTGCAGCT TCTCAGA-3′ and the reverse primer 5′-ACAAAGTCTGGCCATAG GAC-3′ according to the published procedure (37). The PCR products were sequenced on an ABI Prism 3100 automated DNA sequencer using a BigDye 03 Terminator sequencing standards kit (Applied Biosystems, Foster City, CA). The size of each *HMOX1* gene (GT)*n* repeat was calculated using GeneScan Analysis software (PE Applied Biosystems). The number of *HMOX1* (GT)*n* repeats in the DNA strands was determined, and the frequency of repeats in patients was plotted. Assuming a codominant (additive) trait model, the *HMOX1* genotypes were defined by the average length of (GT)*n* repeats. The average length of the *HMOX1* gene promoter (GT)*n* was calculated for each patient.

Plasma measurements. The plasma levels of interleukin-6 (IL-6), IL-10, and tumor necrosis factor alpha (TNFα) were measured using a cytometric bead array system (BD Biosciences Pharmingen, Franklin Lakes, NJ) according to the manufacturer's protocol. All of the samples were run in a single assay in the main laboratory at the Centro de Pesquisas Gonçalo Moniz, Bahia, Brazil. The plasma levels of HO-1 (Assay Designs, Ann Arbor, MI), Hp (GenWay Biotech, San Diego, CA), and sCD163 (BD Pharmingen, Franklin Lakes, NJ) were measured by enzyme-linked immunosorbent assay. Total heme levels were measured using a chromogenic assay according to the manufacturer's instructions (BioAssay Systems, Hayward, CA). The 413-nm and 576-nm UV-visible spectra for the plasma samples were taken with a Nanodrop apparatus to discriminate total heme from non-hemoglobin-bound heme (free heme), as previously described (68). The plasma measurements of aspartate aminotransferase (AST), alanine aminotransaminase (ALT), total bilirubin, hemoglobin, creatinine, fibrinogen, and C-reactive protein (CRP) were performed at the Federal University of Bahia and Faculdade São Lucas, Brazil.

Score-based laboratory assessment of clinical severity of malaria. To infer the degree of systemic inflammation and liver damage during malaria, we used two previously published score systems (4): the hepatic inflammatory (HI) score and the hepatic inflammatory parasitic (HIP) score. To determine the scores, the AST, ALT, fibrinogen, CRP, total bilirubin, and parasitemia levels were estimated in 580 people. This sample consisted of 183 noninfected, 195 symptomatic, and 202 asymptomatic individuals from the same area of endemicity in which the current study was performed. Receiver operator characteristic (ROC) curves were calculated for each parameter with the aim of identifying the best cutoff values to use to differentiate between those individuals with symptomatic and those with asymptomatic malaria with the highest sensitivity and specificity and the highest likelihood ratio (4). One point was given for each parameter that was above the established cutoff, with scores ranging from 0 to 6 and from 0 to 5, including or excluding parasitemia, for HIP and HI, respectively. The individuals presenting with higher scores also referred to severe headaches, fatigue and asthenia, hypotension, and hyperthermia more frequently than those with lower scores (4), implying that higher score values are generally associated with an increased clinical severity of malaria.

Statistical analyses. A chi-squared test was applied to evaluate the association between the following qualitative variables within the patient malaria groups (symptomatic, asymptomatic, and noninfected): *Hp* genotypes/alleles and short and long (<30 and ≥30 GT repeats, respectively) *HMOX1* gene polymorphisms. The plasma levels of Hp, heme, and sCD163 and the *HMOX1* gene (GT)*n* repetitions were compared between groups using a nonparametric Kruskal-Wallis test with Dunn's multiple comparisons. These tests were also used to analyze the difference between *Hp* genotypes/alleles with Hp levels and the association between sCD163, *HMOX1* GT repeat, HO-1, and Hp with the HI/HIP scores. A univariate linear regression analysis was performed to assess the associations between *Hp* alleles/genotypes or *HMOX1* gene polymorphisms and symptomatic malaria. The Mann-Whitney test was used to compare differences in plasma Hp levels, plasma sCD163 levels, and HI/HIP scores between individuals with

TABLE 1 Baseline characteristics of the individuals enrolled in the study

Characteristic	Result by subject group			
	Symptomatic malaria (n = 78)	Asymptomatic malaria (n = 106)	Noninfected (n = 80)	P value
No. (%) male	39 (50.00)	47 (44.34)	37 (46.25)	0.7468[a]
Median (IQR[b]) age (yr)	36 (27.75–50)	42 (32–49)	35 (25.25–45)	0.0307[c]
Median (IQR) no. of previous malaria infection episodes	6.5 (1–13)	15 (12–19)	12.5 (6.25–17)	<0.0001[c]
No. (%) of individuals residing in the area for the following no. of yr:				0.0011[a]
≤2	26 (33.33)	24 (22.64)	19 (23.75)	
3 to 10	17 (21.80)	7 (6.60)	19 (23.75)	
>10	35 (44.87)	75 (70.76)	42 (52.50)	

[a] Categorized variables were compared using a chi-square test.
[b] IQR, interquartile range.
[c] Ordinal variables were compared using the Kruskal-Wallis test with Dunn's multiple-comparison test.

short or long *HMOX1* gene (GT)n repeats. The correlations between Hp levels with parasitemia, ALT, and heme levels were analyzed by Spearman's correlation test. This test was also used to estimate the significance of the correlation between *HMOX1* (GT)n repetitions and sCD163, Hp, C-reactive protein, and creatinine levels and HI and HIP scores. ROC curves were used to evaluate the power of sCD163 to discriminate the individuals with symptomatic malaria from those not infected with *Plasmodium* or those with asymptomatic malaria. Within all comparisons, the differences in which P was <0.05 were considered statistically significant. The statistical analyses were performed using GraphPad Prism (version 5.0b) software (GraphPad Software, San Diego, CA).

RESULTS

Baseline characteristics. The majority of the individuals studied were female (53.40%) adults (age, 39.95 years; standard deviation [SD], ±14.99) who had lived in the area of endemicity for more than 6 months (73.86% more than 3 years and 57.58% more than 10 years). The patients infected with the malaria parasite (n = 184) were approximately the same age (39.68 years; SD, ±14.83) and included more females (53.26%) and individuals who had lived in the area of endemicity for long periods of time (72.83% more than 3 years and 59.78% more than 10 years). The majority of the individuals infected with a *Plasmodium* sp. were asymptomatic at the time of the study and during the 30 days of follow-up (n = 106). The individuals with asymptomatic malaria were also older and had lived in the area of endemicity for longer periods than those with symptomatic infections (Table 1). Most of the patients with symptomatic malaria presented with uncomplicated disease, with only 5 cases of severe malaria present. *Plasmodium vivax* was the main malarial agent (93.40% of symptomatic malaria cases and 91.03% of asymptomatic cases), and *Plasmodium falciparum* was detected in the rest of the malaria cases.

Haptoglobin genotype. The *Hp2.1S* genotype was the most frequently detected genotype in all of the different study groups, representing 41.25% (n = 33) of the noninfected individuals, 32.05% (n = 25) of the asymptomatic malaria cases, and 37.74% (n = 40) of those with symptomatic infections (chi-square P = 0.035). The *Hp1S* allele was observed more frequently in asymptomatic cases and corresponded to 44.81% (n = 95) of the Hp alleles seen in this group. The *Hp2* allele was the most commonly observed allele in both the noninfected and symptomatic malaria cases, representing 45.00% (n = 72) and 48.72% (n = 76) of the alleles in each group, respectively (Fig. 1A and B). Remarkably, the *Hp2* allele was associated with a higher risk of developing symptomatic malaria than asymptomatic malaria upon *Plasmodium* infection (odds ratio [OR] = 1.666, P =

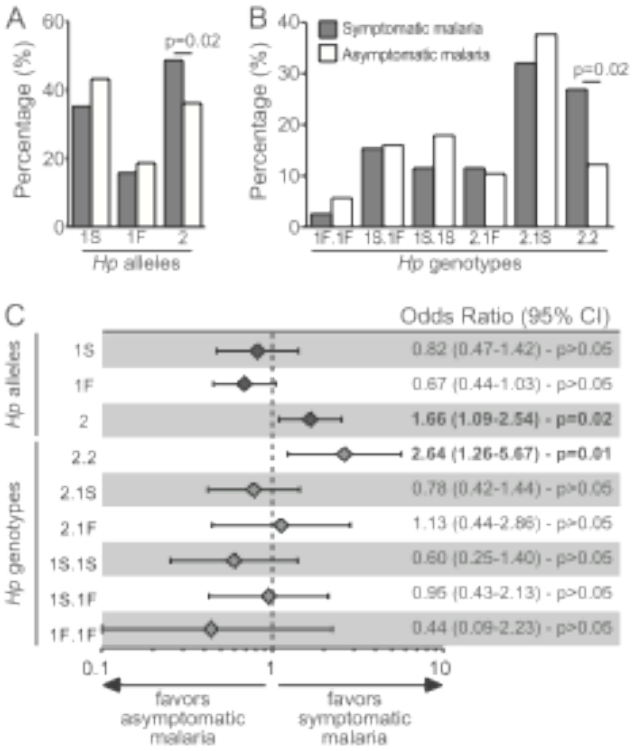


FIG 1 Haptoglobin genetic profiles influence malaria susceptibility. We studied 80 uninfected healthy subjects, 106 subjects with asymptomatic malaria, and 78 subjects with symptomatic *Plasmodium* infection. All of the study subjects were from the Brazilian Amazon. (A) Percentage of individuals with symptomatic (gray bars) or asymptomatic (white bars) malaria carrying the different haptoglobin (*Hp*) alleles, with both homozygous and heterozygous individuals considered for each allele. (B) Percentage of individuals with symptomatic (gray bars) or asymptomatic (white bars) malaria carrying the different *Hp* genotypes. The differences between the groups illustrated in panels A and B were compared using Fisher's exact test. (C) Univariate linear regression analyses of the different *Hp* alleles and genotypes were performed to estimate malaria susceptibility. The odds ratios, respective 95% confidence intervals (95% CIs), and P values are shown in each panel.

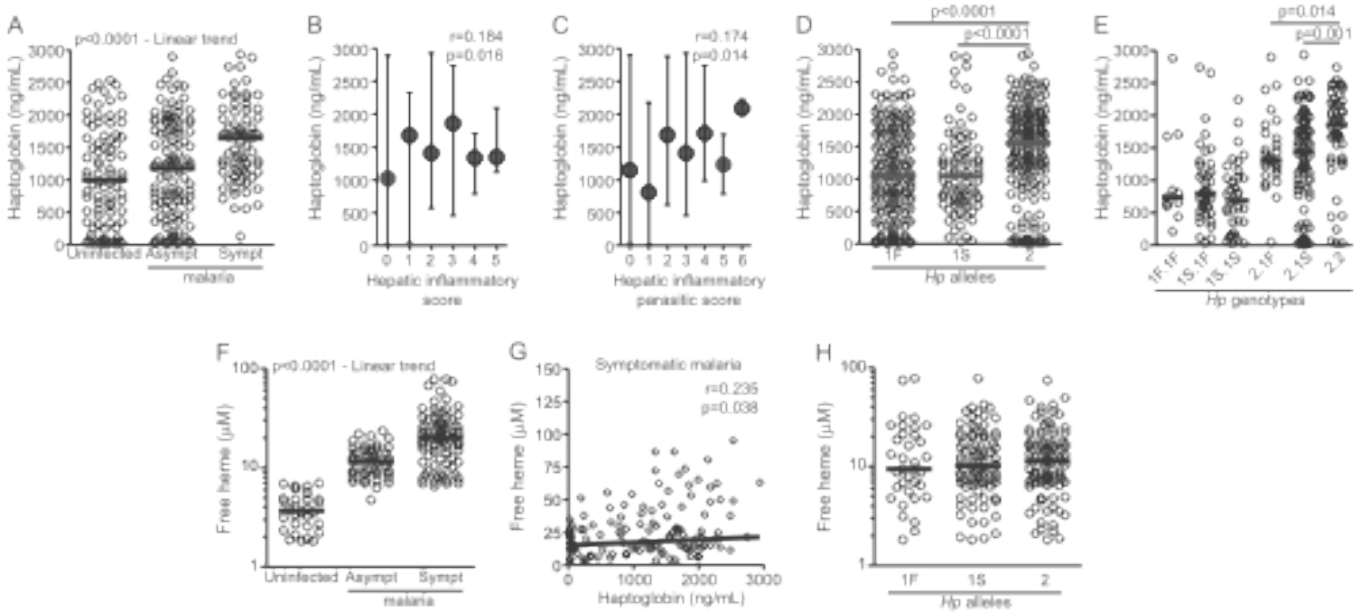


FIG 2 Associations between the systemic levels of haptoglobin and total heme and malaria susceptibility. The plasma concentrations of Hp were measured in the subjects referred to in the legend to Fig. 1. Each symbol represents a single patient, and the lines represent medians. The systemic Hp levels in the different clinical outcome groups were compared (A) and correlated with the degree of hepatic damage and inflammation, as evaluated by the severity scores described in Materials and Methods (B and C). The Hp levels in individuals with different *Hp* alleles (D) or genotypes (E) were also compared. The systemic levels of free heme were compared in patients with different malaria outcomes (F), correlated with the amounts of Hp in the plasma (G), and then compared among individuals with various *Hp* alleles (H). The data were compared using the Mann-Whitney test (comparisons between two groups), the Kruskal-Wallis test with Dunn's multiple comparisons, or linear trend analysis (comparisons between more than two groups). In panels B, C, and G, the data were analyzed using Spearman's rank correlation test. *P* values are shown in each graph.

0.0228; Fig. 1C). That is, individuals who carry the *Hp2* allele have a greater chance of developing symptoms once they are infected by *Plasmodium*. In addition, the *Plasmodium*-infected individuals with the homozygous *Hp2.2* genotype had an even greater chance of developing malaria symptoms (OR = 2.636, $P = 0.0193$; Fig. 1C). Thus, these findings show that the *Hp2* allele and the *Hp2.2* genotype are strongly associated with an increased susceptibility to development of malaria-related symptoms upon *Plasmodium* infection.

We then tested whether the different *Hp* genotypes are indeed associated with different systemic concentrations of Hp. Notably, increased plasma levels of Hp were directly associated with the clinical presentation of malaria, with symptomatic individuals exhibiting higher Hp levels than individuals with asymptomatic infection or noninfected individuals (Fig. 2A). By using a previously reported, laboratory-based score to standardize a reproducible evaluation of the severity of *P. vivax* infection (4), we found that the plasma haptoglobin values were indeed positively correlated with the hepatic inflammatory HI and HIP scores ($r = 0.184$ and $P = 0.016$ for HI and $r = 0.174$ and $P = 0.014$ for HIP; Fig. 2B and C). Thus, the amount of Hp in the circulation increases during symptomatic infection, and individuals with inflammation-associated liver damage present higher levels of Hp. Notably, those individuals who were homozygous or heterozygous for the *Hp2* allele displayed augmented plasma concentrations of Hp compared with carriers of the *Hp1F* and *Hp1S* alleles (Fig. 2D). Moreover, of all the *Hp2*-containing genotypes, individuals with the *Hp2.2* genotype exhibited the highest systemic Hp levels (Fig. 2E). Interestingly, the *Hp2.2* genotype has been linked to a reduced Hp binding affinity for cell-free Hb (39). Indeed, in the present study, the individuals with symptomatic malaria were more likely to have the *Hp2.2* genotype and higher concentrations of free heme in the plasma than those with asymptomatic malaria or uninfected individuals (Fig. 2F). The plasma Hp levels were also positively correlated with the amount of free heme in the plasma ($r = 0.2350$, $P = 0.038$; Fig. 2G). These results suggest that individuals with the *Hp2* allele or the *Hp2.2* genotype produce more Hp protein, probably to compensate for the lower affinity that this Hp has for free Hb in the circulation. Consequently, individuals carrying the *Hp2* allele need to produce more Hp protein to have the same amount of free heme as individuals with the other *Hp* alleles (Fig. 2H). Thus, the Hb binding affinity of Hp seems to be more important than their Hp levels in determining an individual's susceptibility to symptomatic malaria.

***HMOX1* microsatellite polymorphism.** Free heme is a potent inducer of HO-1 (14), and we found that symptomatic patients who presented with high levels of circulating free heme also displayed increased systemic concentrations of HO-1 compared with noninfected individuals or those with asymptomatic infections (Fig. 3A). In addition, the patients with both symptomatic and asymptomatic malaria had longer (GT)*n* dinucleotide repeats than noninfected volunteers (29.90 ± 3.225, 28.74 ± 2.987, and 28.01 ± 2.868 repeats, respectively; $P < 0.001$; Fig. 3B). The classification of the *HMOX1* polymorphism based on the number of GT repeats varies among studies and depends on the frequency peaks of the repeats found in each study sample (21, 37, 61). In our study, the number of (GT)*n* repeats ranged from 19 to 40, with three frequency peaks at 28, 29, and 30 repeats (Fig. 3C). The inducibility of the *HO-1* gene promoter is known to be negatively correlated with the number of GT repeats (65), and we adapted

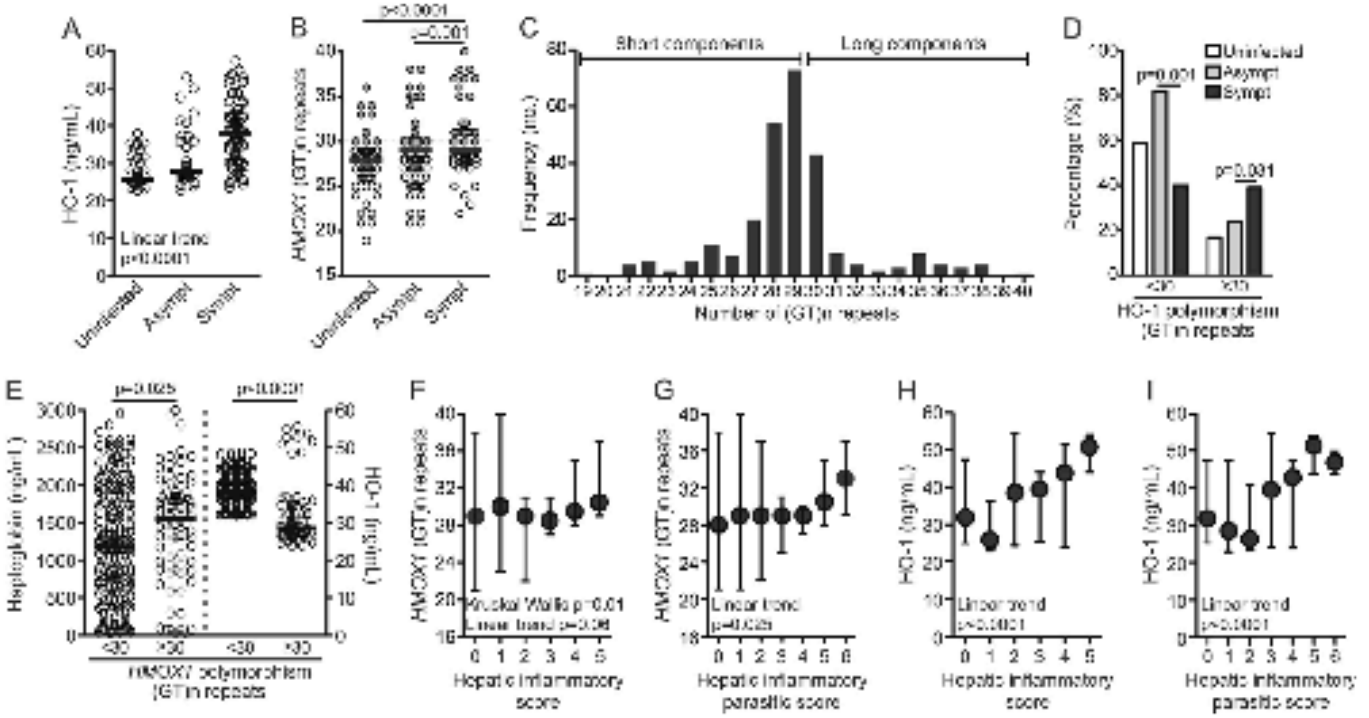


FIG 3 *HMOX1* gene polymorphisms influence susceptibility to malaria. (A) The plasma HO-1 concentrations in noninfected individuals and those with asymptomatic or symptomatic malaria were compared. (B) The numbers of GT repeats in the *HMOX1* gene in noninfected individuals and those presenting with asymptomatic or symptomatic malaria were also compared. Gray dots represent individuals carrying ≥30 GT repeats in the *HMOX1* gene. (C) Frequency of the different number of (GT)*n* repeats in the study population. (D) Percentage of noninfected individuals (white bars) and individuals with asymptomatic (gray bars) or symptomatic (black bars) malaria carrying the short or long (GT)*n* repeats in the *HMOX1* gene. (E) Plasma levels of Hp (left) and HO-1 (right) in individuals with short or long (GT)*n* repeats in the *HMOX1* gene (data were compared using a Mann-Whitney test). (F and G) The associations between the number of (GT)*n* repeats in the *HMOX1* gene and the degree of liver damage or disease severity were estimated by the hepatic inflammatory and hepatic inflammatory parasitic scores. (H and I) Plasma HO-1 concentrations in relation to the malaria severity scores. In panels F to I, the symbols represent the median values and the whiskers represent maximum and minimum values. The differences between the groups illustrated in panel D were compared using a chi-square exact test and Fisher's exact test (only the *P* values from Fisher's exact test are shown). The data from the other panels were compared using the Kruskal-Wallis test with Dunn's multiple comparisons or linear trend analysis. *P* values are shown in each panel.

previous classifications of the *HMOX1* gene polymorphisms (69) to stratify the classification into two categories: a short form (<30 GT repeats) and a long form (≥30 GT repeats).

The individuals carrying a short form of the *HMOX1* gene polymorphism were more likely to present with asymptomatic malaria, whereas those with longer repeats were mostly symptomatic (Fig. 3D). Thus, long *HMOX1* GT repeats are associated with an increased susceptibility to develop symptoms upon *Plasmodium* infection. In agreement with a previous study (24), we found that plasma HO-1 levels were consistently lower in the patients who carry the long form of the *HMOX1* gene polymorphism than the patients with the short GT form (Fig. 3E). In contrast, the patients with the long GT form displayed higher levels of Hp (Fig. 3E), arguing that those individuals who have the long form and low concentrations of HO-1 also have higher levels of Hp, possibly as a regulatory mechanism against hemolysis. Although the symptomatic patients presented with higher overall HO-1 levels than uninfected individuals or those with asymptomatic malaria, the patients with longer (GT)*n* repeats were more likely to have symptomatic malaria and relatively lower levels of HO-1 than the patients with long repeats (Fig. 3A and E). There was no association between the systemic concentrations of HO-1 and the *Hp* genotypes (data not shown).

The individuals with ≥30 GT repeats had greater susceptibility for developing symptomatic malaria than did the individuals with <30 repeats (OR = 3.35, confidence interval [CI] = 1.91 to 5.88, $P = 0.0002$). Intriguingly, we found an association between the severity scores and the number of GT repeats (Fig. 3F and G) and the HO-1 plasma values (Fig. 3H and I). Thus, the patients with the long form of the *HMOX1* gene polymorphism displayed higher severity scores than did those carrying shorter *HMOX1* GT forms. Despite carrying the long form of the *HMOX1* polymorphism more frequently, the symptomatic malaria patients who presented with increased inflammatory damage had higher systemic concentrations of the HO-1 protein. This finding suggests that higher HO-1 levels are a counterregulatory response to inflammation, despite the fact that this genetic background is associated with a lower *HMOX1* induction. Thus, these observations suggest that, like the *Hp* genotype, the long forms of the *HMOX1* gene polymorphism can increase susceptibility to malaria, but with a different level of complexity.

Systemic levels of the Hp receptor sCD163. The binding of Hp to the CD163 receptor on the surface of monocytes or macrophages leads to the removal of the Hp complexes formed by cell-free Hb and haptoglobin (39). The soluble form of the CD163 receptor, sCD163, is a surrogate marker of systemic inflammation, and increased levels of this marker are correlated with a poor prognosis in a number of pathological conditions. In this study,

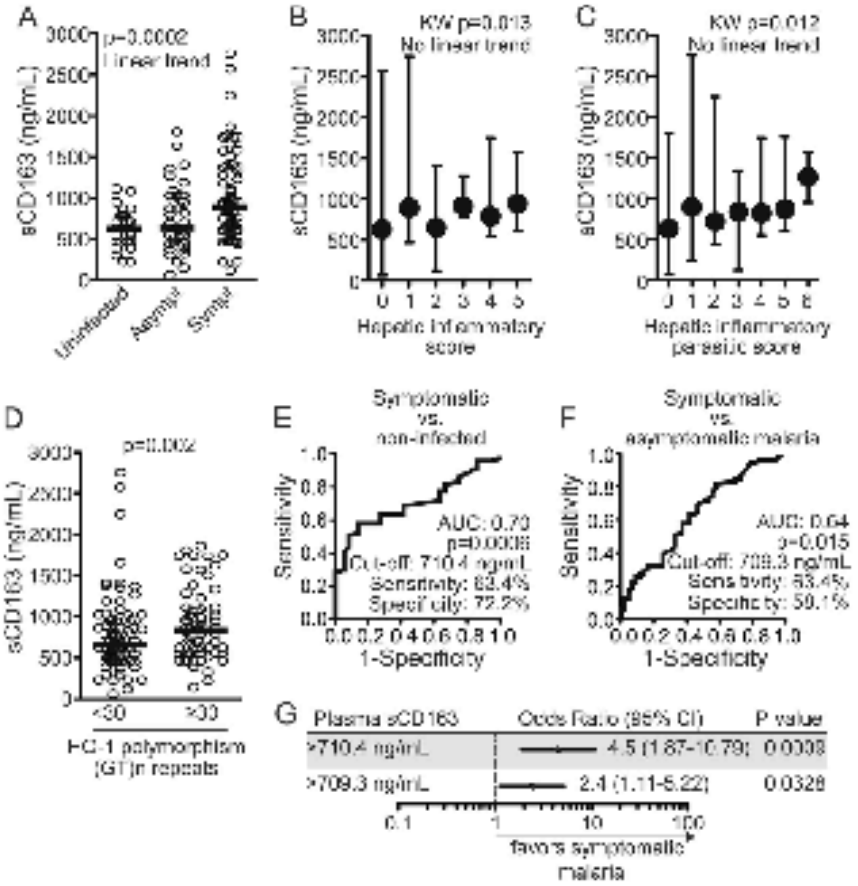


FIG 4 Soluble CD163 and susceptibility to malaria. (A) The systemic concentration of sCD163 was quantified in all subjects and correlated with different malaria outcomes. (B and C) The systemic levels of sCD163 were tested for associations with the hepatic inflammatory scores using Kruskal-Wallis tests with linear trend posttest analysis. (D) The plasma sCD163 levels in individuals with short and long (GT)*n* repeats in the *HMOX1* gene were compared. The data were compared using a Mann-Whitney test. ROC curve analyses were performed to depict the power of sCD163 to discriminate individuals with symptomatic malaria from those not infected with *Plasmodium* (E) or from those with asymptomatic malaria (F). AUC, area under the curve. The cutoff values for sCD163 levels were established by C statistics and are shown in each graph. (G) A univariate logistic regression analysis was performed to test associations between sCD163 concentrations above the established cutoff values, and the sCD163 concentrations were used to determine the chance of developing malaria-related symptoms by comparison with the sCD163 concentrations for the uninfected individuals (first line; sCD163 cutoff, 710.4 ng/ml) or individuals with asymptomatic malaria (second line; sCD163 cutoff, 709.3 ng/ml). The odds ratios, respective 95% confidence intervals (95% CIs), and *P* values are shown in each panel.

the individuals with symptomatic malaria had higher systemic sCD163 levels than did those with symptomless infection or noninfected individuals (Fig. 4A). In addition, the sCD163 levels were positively correlated with plasma Hp levels ($r = 0.2477$, $P = 0.0028$), and there were no associations between sCD163 concentrations and the *Hp* genotypes of the patients (data not shown). The sCD163 levels were also correlated with the plasma concentrations of TNF ($r = 0.2095$, $P = 0.0101$), IL-6 ($r = 0.1991$, $P = 0.0146$), C-reactive protein ($r = 0.2618$, $P = 0.0012$), and creatinine ($r = 0.2693$, $P = 0.0009$). The laboratory scores that suggest malaria severity (HI and HIP) were positively correlated with sCD163 (HI, $r = 0.1831$ and $P = 0.0249$; HIP, $r = 0.1726$ and $P = 0.0353$; Fig. 4B and C). These findings indicate that sCD163 is indeed associated with systemic inflammation and malaria symptomatology.

Interestingly, the systemic sCD163 concentrations were positively correlated with the number of (GT)*n* dinucleotide repetitions in the *HMOX1* gene polymorphisms ($r = 0.1646$, $P = 0.0441$), and individuals with ≥30 GT repeats had higher levels of sCD163 than did those with <30 GT repeats (Fig. 4D). Although we found a positive association between *HMOX1* gene polymorphisms and systemic sCD163 levels, we could not detect a correlation between this soluble marker and the plasma concentrations of HO-1 or free heme levels (data not shown). This finding supports the idea that sCD163 plays a role in the onset of malaria symptoms, and this effect may not be directly associated with the genetic profiles of the *Hp* and *HMOX1* genes. Indeed, plasma sCD163 levels could discriminate those patients with symptomatic malaria from noninfected individuals (Fig. 4E) and those with asymptomatic malaria (Fig. 4F). Univariate logistic regression analysis confirmed the association between high sCD163 levels and a susceptibility to development of malaria symptoms (Fig. 4G). Interestingly, the individuals who were carriers of the *Hp2.2* and long *HMOX1* (GT)*n* repeats and exhibited high systemic concentrations of sCD163 were more likely to have symptomatic malaria than the individuals without any of the three potential risk factors, e.g., *Hp1.1* or *Hp2.1* carriers with short *HMOX1* (GT)*n* repeats and low systemic concentrations of sCD163 (chi-square $P = 0.0055$). These data indicate that a combined contribution of

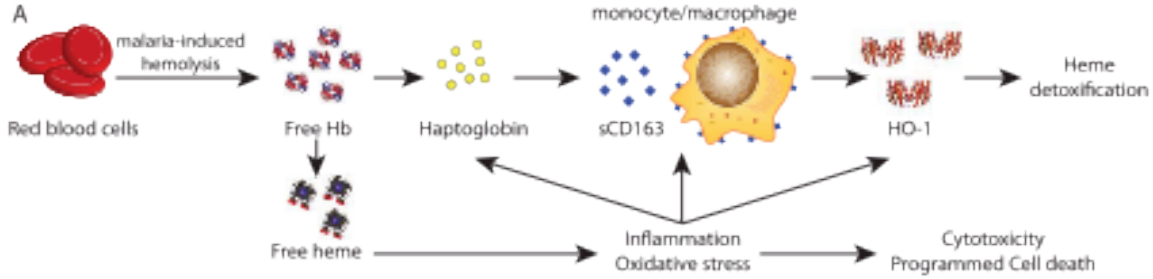
these three factors involved in the detoxification of free heme (Hp, sCD163, and HO-1) contributes to the determination of malaria susceptibility.

DISCUSSION

To our knowledge, the present study is the first to simultaneously assess genetic alterations and the plasma concentrations of different key components of the detoxification of free heme in malaria patients. In addition, we are the first to report an association between the *Hp* and *HMOX1* genes and sCD163 levels and susceptibility to disease in the context of *Plasmodium vivax* infection. From a clinical standpoint, the infections caused by *P. vivax* and *P. falciparum* are unequal and happen as the result of different host immune and inflammatory responses. The malaria caused by *P. falciparum* is more frequently associated with acute life-threatening complications, whereas vivax malaria is usually mild and nonlethal. These differences may be associated with the differential induction of the host's defense mechanisms for circumventing the deleterious effects of heme. Our study does not address these differences, and additional epidemiological and mechanistic studies are necessary to answer this question. Our results demonstrate that individuals with the *Hp2.2* phenotype have a higher risk of developing symptomatic (as opposed to asymptomatic) malaria upon *Plasmodium* infection. The presence of the *Hp2.2* genotype has been associated with an increase in redox-active iron and oxidative stress compared with the presence of the *Hp1.1* genotype (9, 47). Moreover, the Hb-*Hp2.2* complex, but not other *Hp1* complexes, can be internalized by monocytes and stimulate the release of proinflammatory cytokines (57). Indeed, the *Hp2.2* phenotype has been associated with susceptibility to several inflammatory conditions, including malaria (11, 20, 22, 28, 36, 55, 58). Haptoglobin is considered an acute-phase protein that increases 2- to 4-fold during the response to acute inflammation (35). We observed that heme and Hp levels were higher in those individuals with symptomatic malaria than those with symptomless infection or those not infected with *Plasmodium*. Interestingly, the subjects with the *Hp2.2* genotype presented with augmented systemic concentrations of Hp compared with those carrying the *Hp1* allele. Although acute and severe hemolysis will always lead to a reduction in Hp clearance, as seen in severe malaria (32), the response to chronic or low-level hemolysis, which is commonly seen in mild vivax malaria, is difficult to predict. Evidence suggests that, unlike the Hb–*Hp1-1* complex, the Hb-*Hp2.2* complex can stimulate the release of IL-10 and IL-6 (31) and that IL-6 expression increases the synthesis of Hp. It is also possible that the higher Hp production in individuals with the *Hp2* allele acts as a compensatory mechanism for the lower affinity of this Hp for cell-free Hb (39).

We describe herein that subjects with the long form (≥30 GT repeats) of the *HMOX1* gene polymorphism have greater susceptibility to developing symptomatic malaria than individuals with the short form (<30 repeats), suggesting that the *HMOX1* gene polymorphism is involved in susceptibility to *Plasmodium* infection. The individuals who carried longer (GT)*n* dinucleotide repeats and had symptomatic infections also had higher HI and HIP inflammatory scores, suggesting an association between the *HMOX1* gene and the control of inflammation. Sambo et al. (59) found that shorter GT repeats in the *HMOX1* gene are associated with patients with cerebral malaria as opposed to patients with uncomplicated malaria or a noninfected control group. However, this difference was not seen when comparing the group of cerebral malaria patients with patients exhibiting other severe forms of malaria. The malaria patients in this study were mostly infected by *P. vivax* and more frequently exhibited the noncomplicated forms of the disease. Furthermore, another study also reported an association between short (GT)*n* dinucleotide repetitions in the *HMOX1* gene and human cerebral malaria caused by *P. falciparum*, suggesting that higher expression of HO-1 is detrimental for malaria (63). Nevertheless, increased concentrations of HO-1 have been strongly associated with protection against malaria in mice. This protection mainly occurs through the production of carbon monoxide (CO) gas, which binds to cell-free Hb with very high affinity. This interaction results in the formation of carboxyhemoglobin, which prevents heme release and an increase in intravascular free heme (26, 53).

In general, we found that the individuals with symptomatic malaria had higher plasma HO-1 concentrations than did individuals with symptomless infection or noninfected subjects. HO-1 is an intracellular enzyme, and the source of this molecule in the plasma is unclear. A reasonable explanation would be the release of HO-1 after cellular lysis during inflammation. Additionally, elevated plasma HO-1 levels have been reported with other diseases, such as vasculitis in Henoch-Schonlein purpura (19), hemophagocytic syndrome from hematological disorders (38, 49), type 2 diabetes (13), and prostate cancer (16). We speculate that HO-1 could play an anti-inflammatory role by degrading heme, which would dictate the severity of malaria. However, this effect remains to be established. Some studies using experimental models of malaria suggest that deleting the *HMOX1* gene or pharmacologically inhibiting HO-1 activity in mice accounts for the pathogenesis of malaria, as these mice will not have the enzyme responsible for the detoxification of deleterious free heme (25). In contrast, our results show that individuals with symptomatic malaria have higher plasma HO-1 levels than do those with asymptomatic infection. In symptomatic individuals, the increased amounts of free heme and the cytokine storm that is associated with inflammation could be inducing increased levels of HO-1 as a counterregulatory response, especially considering the fact that *HMOX1* gene expression is highly inducible by heme (64). Nevertheless, we have also found a group of symptomatic subjects who were more likely to be carriers of long (GT)*n* dinucleotide repeats in the *HMOX1* gene microsatellite and had lower systemic levels of HO-1. In particular, those individuals with the long form of the *HMOX1* polymorphism are generally those who have lower expression of the enzyme as a result of the genetic factor. In our study, the individuals with symptomatic malaria who presented with low expression of HO-1 were also the ones who carried the long form of the *HMOX1* (GT)*n* polymorphism. In these cases, the genetic factor is probably preventing the counterregulatory increase in HO-1. Furthermore, malaria symptomatology may be associated with either increased or decreased expression of HO-1. High HO-1 levels may result from a counterregulatory response to infection and the cytokine storm and can lead to the increased synthesis of iron (a heme catabolism product), which can also be harmful to humans (63). Low HO-1 levels can result from a genetic component that results in a higher concentration of deleterious free heme in the circulation and is associated with symptomatic malaria (25, 26). Our results argue that both high and low levels of HO-1 may be associated with a greater chance of developing symptoms after *Plasmodium* infection. Our group is cur-



B

	Free heme levels	Haptoglobin levels	*Hp* genetic profile	sCD163 levels	HO-1 levels	*HMOX1* GT repeats	Inflammatory scores
Asymptomatic malaria	↑	↑	*Hp1S* allele	unchanged	unchanged	short	low
Symptomatic malaria	↑↑	↑↑	*Hp2* allele and *Hp2.2* genotype	↑↑	↓ or ↑↑	long	high

FIG 5 Heme metabolism and malaria outcomes. The diagram illustrates a summary of the major mechanisms that are triggered by hemolysis during malaria. (A) Under homeostatic conditions, the free Hb that is released by dead red blood cells is rapidly scavenged by haptoglobin, and this molecular complex is removed from the circulation by the haptoglobin-Hb receptor CD163 on the surface of monocytes and macrophages. The Hb is processed inside these cells in an event that releases heme, which is further metabolized by the antioxidant enzyme HO-1. During an acute malarial attack, there is an accumulation of circulating free hemoglobin that is not compensated for by the amount of Hp available in the blood. The excess of free Hb is then oxidized by free radicals, releasing free heme. Free heme is very toxic to the cells and induces inflammation, macrophage activation, and oxidative stress. The host homeostatic responses triggered by heme include the induction of Hp, CD163, and HO-1. (B) We found that the intensity of the malaria-related symptoms is associated with the levels of circulating free heme. The individuals who developed symptoms after *Plasmodium* infection exhibited higher levels of Hp, soluble CD163, and HO-1. Nevertheless, this counterregulatory response is not sufficient to reduce the amount of free heme in the plasma, which also might explain the higher inflammatory scores estimated in symptomatic patients. These susceptible individuals carried the *Hp2* allele or the *Hp2.2* genotype more frequently than did other individuals. In addition, they more frequently carried long GT repeats in the *HMOX1* polymorphism, which are paradoxically associated with a relatively lower plasma HO-1 concentration. The individuals with clinical immunity against malaria who remained asymptomatic upon *Plasmodium* infection tended to carry the *Hp1S* allele and have short GT repeats in the *HMOX1* polymorphism more frequently than the symptomatic individuals. These protected individuals still had modest elevations in the levels of free heme and haptoglobin, with no differences in the concentrations of sCD163 and HO-1 compared with noninfected individuals.

rently performing mechanistic studies to better understand the effects of HO-1 in human malaria.

This study revealed that plasma sCD163 levels gradually increased in correlation with the severity of the malaria infection. sCD163 has been identified to be an anti-inflammatory mediator that inhibits human T-lymphocyte activation and proliferation, and the binding of Hb-Hp complexes to sCD163 has been shown to suppress the supply of heme iron that is available to hemolytic bacteria (34, 67). Interestingly, several inflammatory processes are associated with elevated levels of sCD163 (18, 29, 33, 45, 46, 50–52, 60, 66), including falciparum malaria (41). Therefore, because symptomatic malaria is associated with a higher inflammatory response, the increased sCD163 concentrations in the symptomatic group probably serve as a counterregulatory mechanism against inflammation. Consistent with the inflammatory response seen during malaria infection, our results found a positive correlation between the levels of sCD163, TNF-α, and acute-phase proteins such as C-reactive protein and Hp. Indeed, sCD163 levels have already been positively correlated with TNF-α levels in falciparum malaria (41) and C-reactive protein in diabetes (50). TNF-α is able to induce the hepatic synthesis of Hp and regulates the expression of CD163 in monocytes and macrophages (41). IL-6 and IL-10 stimulate the expression of membrane-bound CD163 and have been positively correlated with sCD163 levels (17, 62). In this study, sCD163 levels were correlated with IL-6 levels; however, sCD163 levels did not correlate with IL-10 levels. Furthermore, sCD163 was positively correlated with HI and HIP scores, confirming the relationship between this molecule and inflammation in malaria.

Studies that simultaneously evaluate the different steps of the heme detoxification process may help to explain some of the controversies surrounding the effects of alterations in specific elements of this pathway. We have demonstrated that the individuals carrying the *Hp2.2* genotype and the longer *HMOX1* gene (GT)*n* dinucleotide repeats who also presented with high systemic concentrations of sCD163 have a greater susceptibility to developing clinical malaria than do those without these three potential risk factors. As shown in other studies (10–12, 15, 22, 23, 40, 41, 48, 56, 63) as well as in our work, genetic alterations in the *HMOX1* and *Hp* genes and changes in sCD163 levels are all important elements during *Plasmodium* infection. A summary of the determinants involved in heme metabolism and our major findings is depicted in Fig. 5. The majority of the malaria cases in our study were caused by *P. vivax*, which limits our ability to compare our results with most of the findings in the current literature, which has focused on falciparum malaria. Research aimed at understanding the key factors involved in the immunopathogenesis of susceptibility to vivax malaria has been relatively neglected and made a low priority. There are clear similarities in the diseases caused by *P. falciparum* and *P. vivax*. Both infections cause hemolysis, for example. However, there are important and well-described differences between *P. vivax* and *P. falciparum* that result in different parasitemia thresholds for triggering severe malaria (2). The different clinical outcomes of these diseases make it important to expand the studies investigating the factors that are associated with the susceptibility to infection and disease severity in *P. vivax* malaria. Thus, studies assessing larger populations of *P. vivax*-infected individuals are needed to clarify the roles that sCD163,

Hp, and HO-1 play in heme metabolism during human malaria infections. The validation of the results presented in the current study may provide new resources for the development of future targeted therapies that could aid in reducing malaria severity.

ACKNOWLEDGMENTS

We thank Sebastião Souza-Neto, Antonio Reis-Filho, Jorge Clarêncio, Imbroinise Rafaelle-Netto, Jaqueline França-Costa, Jorge Tolentino, and Adorielze Leite for their technical and logistical support. We also thank Aldina Barral, Erney Camargo, and Luis Camargo for their critical discussions and revision of the data.

We declare that we have no conflicts of interest.

REFERENCES

1. **Andrade BB, et al.** 2010. Heme impairs prostaglandin E2 and TGF-beta production by human mononuclear cells via Cu/Zn superoxide dismutase: insight into the pathogenesis of severe malaria. J. Immunol. **185**: 1196–1204.
2. **Andrade BB, Barral-Netto M.** 2011. Biomarkers for susceptibility to infection and disease severity in human malaria. Mem. Inst. Oswaldo Cruz **106**(Suppl 1):70–78.
3. **Andrade BB, et al.** 2010. Towards a precise test for malaria diagnosis in the Brazilian Amazon: comparison among field microscopy, a rapid diagnostic test, nested PCR, and a computational expert system based on artificial neural networks. Malar. J. **9**:117.
4. **Andrade BB, et al.** 2010. Severe Plasmodium vivax malaria exhibits marked inflammatory imbalance. Malar. J. **9**:13.
5. **Andrade BB, et al.** 2010. Plasma superoxide dismutase-1 as a surrogate marker of vivax malaria severity. PLoS Negl. Trop. Dis. **4**:e650.
6. **Andrade BB, et al.** 2009. Anti-Anopheles darlingi saliva antibodies as marker of Plasmodium vivax infection and clinical immunity in the Brazilian Amazon. Malar. J. **8**:121.
7. **Andrade BB, et al.** 2011. Hepatitis B infection is associated with asymptomatic malaria in the Brazilian Amazon. PLoS One **6**:e19841.
8. **Arredouani M, et al.** 2003. Haptoglobin directly affects T cells and suppresses T helper cell type 2 cytokine release. Immunology **108**:144–151.
9. **Asleh R, Guetta J, Kalet-Litman S, Miller-Lotan R, Levy AP.** 2005. Haptoglobin genotype- and diabetes-dependent differences in iron-mediated oxidative stress in vitro and in vivo. Circ. Res. **96**:435–441.
10. **Atkinson SH, et al.** 2007. The haptoglobin 2-2 genotype is associated with a reduced incidence of Plasmodium falciparum malaria in children on the coast of Kenya. Clin. Infect. Dis. **44**:802–809.
11. **Atkinson SH, et al.** 2006. Seasonal childhood anaemia in West Africa is associated with the haptoglobin 2-2 genotype. PLoS Med. **3**:e172.
12. **Aucan C, Walley AJ, Greenwood BM, Hill AV.** 2002. Haptoglobin genotypes are not associated with resistance to severe malaria in The Gambia. Trans. R. Soc. Trop. Med. Hyg. **96**:327–328.
13. **Bao W, et al.** Plasma heme oxygenase-1 concentration is elevated in individuals with type 2 diabetes mellitus. PLoS One **5**:e12371.
14. **Beschorner R, et al.** 2000. Long-term expression of heme oxygenase-1 (HO-1, HSP-32) following focal cerebral infarctions and traumatic brain injury in humans. Acta Neuropathol. **100**:377–384.
15. **Bienzle U, et al.** 2005. Limited influence of haptoglobin genotypes on severe malaria in Ghanaian children. Trop. Med. Int. Health **10**:668–671.
16. **Blann AD, Balakrishnan B, Ryan P, Lip GY.** Increased levels of plasma haemoxygenase-1 in prostate cancer. Prostate Cancer Prostatic Dis. **14**: 114–117.
17. **Buechler C, et al.** 2000. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. J. Leukoc. Biol. **67**:97–103.
18. **Burdo TH, et al.** 2011. Soluble CD163 made by monocyte/macrophages is a novel marker of HIV activity in early and chronic infection prior to and after anti-retroviral therapy. J. Infect. Dis. **204**:154–163.
19. **Chen T, et al.** 2011. Elevated serum heme oxygenase-1 and insulin-like growth factor-1 levels in patients with Henoch-Schonlein purpura. Rheumatol. Int. **31**:321–326.
20. **Chen YC, et al.** 2011. Haptoglobin polymorphism as a risk factor for chronic kidney disease: a case-control study. Am. J. Nephrol. **33**:510–514.
21. **Chin HJ, et al.** 2009. The heme oxygenase-1 genotype is a risk factor to renal impairment of IgA nephropathy at diagnosis, which is a strong predictor of mortality. J. Korean Med. Sci. **24**(Suppl):S30–S37.
22. **Cox SE, et al.** 2007. Haplotype association between haptoglobin (Hp2) and Hp promoter SNP (A-61C) may explain previous controversy of haptoglobin and malaria protection. PLoS One **2**:e362.
23. **Elagib AA, Kider AO, Akerstrom B, Elbashir MI.** 1998. Association of the haptoglobin phenotype (1-1) with falciparum malaria in Sudan. Trans. R. Soc. Trop. Med. Hyg. **92**:309–311.
24. **Exner M, Minar E, Wagner O, Schillinger M.** 2004. The role of heme oxygenase-1 promoter polymorphisms in human disease. Free Radic. Biol. Med. **37**:1097–1104.
25. **Ferreira A, Balla J, Jeney V, Balla G, Soares MP.** 2008. A central role for free heme in the pathogenesis of severe malaria: the missing link? J. Mol. Med. (Berl.) **86**:1097–1111.
26. **Ferreira A, et al.** 2011. Sickle hemoglobin confers tolerance to Plasmodium infection. Cell **145**:398–409.
27. **Francis SE, Sullivan DJ, Jr, Goldberg DE.** 1997. Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. Annu. Rev. Microbiol. **51**:97–123.
28. **Friis H, et al.** 2003. Iron, haptoglobin phenotype, and HIV-1 viral load: a cross-sectional study among pregnant Zimbabwean women. J. Acquir. Immune Defic. Syndr. **33**:74–81.
29. **Funding M, et al.** 2005. Soluble CD163 and interleukin-6 are increased in aqueous humour from patients with endothelial rejection of corneal grafts. Acta Ophthalmol. Scand. **83**:234–239.
30. **Gozzelino R, Soares MP.** 2011. Heme sensitization to TNF-mediated programmed cell death. Adv. Exp. Med. Biol. **691**:211–219.
31. **Guetta J, Strauss M, Levy NS, Fahoum L, Levy AP.** 2007. Haptoglobin genotype modulates the balance of Th1/Th2 cytokines produced by macrophages exposed to free hemoglobin. Atherosclerosis **191**:48–53.
32. **Gyan B, et al.** 2002. Elevated levels of nitric oxide and low levels of haptoglobin are associated with severe malarial anaemia in African children. Acta Trop. **83**:133–140.
33. **Hiraoka A, et al.** 2005. Soluble CD163 in patients with liver diseases: very high levels of soluble CD163 in patients with fulminant hepatic failure. J. Gastroenterol. **40**:52–56.
34. **Hogger P, Erpenstein U, Rohdewald P, Sorg C.** 1998. Biochemical characterization of a glucocorticoid-induced membrane protein (RM3/1) in human monocytes and its application as model system for ranking glucocorticoid potency. Pharm. Res. **15**:296–302.
35. **Imrie H, et al.** 2007. Low prevalence of an acute phase response in asymptomatic children from a malaria-endemic area of Papua New Guinea. Am. J. Trop. Med. Hyg. **76**:280–284.
36. **Kasvosve I, et al.** 2000. Haptoglobin polymorphism and mortality in patients with tuberculosis. Int. J. Tuberc. Lung Dis. **4**:771–775.
37. **Kimpara T, et al.** 1997. Microsatellite polymorphism in the human heme oxygenase-1 gene promoter and its application in association studies with Alzheimer and Parkinson disease. Hum. Genet. **100**:145–147.
38. **Kirino Y, et al.** 2005. Increased serum HO-1 in hemophagocytic syndrome and adult-onset Still's disease: use in the differential diagnosis of hyperferritinemia. Arthritis Res. Ther. **7**:R616–R624.
39. **Kristiansen M, et al.** 2001. Identification of the haemoglobin scavenger receptor. Nature **409**:198–201.
40. **Kuesap J, Hirayama K, Kikuchi M, Ruangweerayut R, Na-Bangchang K.** 2010. Study on association between genetic polymorphisms of haem oxygenase-1, tumour necrosis factor, cadmium exposure and malaria pathogenicity and severity. Malar. J. **9**:260.
41. **Kusi KA, et al.** 2008. Levels of soluble CD163 and severity of malaria in children in Ghana. Clin. Vaccine Immunol. **15**:1456–1460.
42. **Langlois MR, Delanghe JR.** 1996. Biological and clinical significance of haptoglobin polymorphism in humans. Clin. Chem. **42**:1589–1600.
43. **Langlois MR, Delanghe JR, De Buyzere ML, Bernard DR, Ouyang J.** 1997. Effect of haptoglobin on the metabolism of vitamin C. Am. J. Clin. Nutr. **66**:606–610.
44. **Langlois MR, et al.** 2000. The haptoglobin 2-2 phenotype affects serum markers of iron status in healthy males. Clin. Chem. **46**:1619–1625.
45. **Levy AP, et al.** 2007. Downregulation of the hemoglobin scavenger receptor in individuals with diabetes and the Hp 2-2 genotype: implications for the response to intraplaque hemorrhage and plaque vulnerability. Circ. Res. **101**:106–110.
46. **Matsushita N, et al.** 2002. Elevated levels of soluble CD163 in sera and fluids from rheumatoid arthritis patients and inhibition of the shedding of CD163 by TIMP-3. Clin. Exp. Immunol. **130**:156–161.
47. **Melamed-Frank M, et al.** 2001. Structure-function analysis of the antioxidant properties of haptoglobin. Blood **98**:3693–3698.

48. **Minang JT, et al.** 2004. Haptoglobin phenotypes and malaria infection in pregnant women at delivery in western Cameroon. Acta Trop. **90**:107–114.
49. **Miyazaki T, et al.** Serum HO-1 is useful to make differential diagnosis of secondary hemophagocytic syndrome from other similar hematological conditions. Int. J. Hematol. **91**:229–237.
50. **Moller HJ, Frikke-Schmidt R, Moestrup SK, Nordestgaard BG, Tybjaerg-Hansen A.** 2011. Serum soluble CD163 predicts risk of type 2 diabetes in the general population. Clin. Chem. **57**:291–297.
51. **Moreno JA, et al.** 2010. Peripheral artery disease is associated with a high CD163/TWEAK plasma ratio. Arterioscler. Thromb. Vasc. Biol. **30**:1253–1262.
52. **Nakayama W, et al.** 2012. Serum levels of soluble CD163 in patients with systemic sclerosis. Rheumatol. Int. **32**:403–407.
53. **Pamplona A, et al.** 2007. Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. Nat. Med. **13**:703–710.
54. **Philippidis P, et al.** 2004. Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxygenase-1 synthesis: antiinflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. Circ. Res. **94**:119–126.
55. **Quaye IK, Ababio G, Amoah AG.** 2006. Haptoglobin 2-2 phenotype is a risk factor for type 2 diabetes in Ghana. J. Atheroscler. Thromb. **13**:90–94.
56. **Quaye IK, et al.** 2000. Haptoglobin 1-1 is associated with susceptibility to severe Plasmodium falciparum malaria. Trans. R. Soc. Trop. Med. Hyg. **94**:216–219.
57. **Rogerson S.** 2006. What is the relationship between haptoglobin, malaria, and anaemia? PLoS Med. **3**:e200.
58. **Ryndel M, et al.** 2010. The haptoglobin 2-2 genotype is associated with carotid atherosclerosis in 64-year old women with established diabetes. Clin. Chim. Acta **411**:500–504.
59. **Sambo MR, et al.** 2010. Transforming growth factor beta 2 and heme oxygenase 1 genes are risk factors for the cerebral malaria syndrome in Angolan children. PLoS One **5**:e11141.
60. **Schaer DJ, et al.** 2005. Soluble hemoglobin-haptoglobin scavenger receptor CD163 as a lineage-specific marker in the reactive hemophagocytic syndrome. Eur. J. Haematol. **74**:6–10.
61. **Sheu CC, et al.** 2009. Heme oxygenase-1 microsatellite polymorphism and haplotypes are associated with the development of acute respiratory distress syndrome. Intensive Care Med. **35**:1343–1351.
62. **Sulahian TH, et al.** 2000. Human monocytes express CD163, which is upregulated by IL-10 and identical to p155. Cytokine **12**:1312–1321.
63. **Takeda M, et al.** 2005. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to cerebral malaria in Myanmar. Jpn. J. Infect. Dis. **58**:268–271.
64. **Tenhunen R, Marver HS, Schmid R.** 1970. The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. J. Lab. Clin. Med. **75**:410–421.
65. **Wagener FA, et al.** 2008. HMOX1 promoter polymorphism modulates the relationship between disease activity and joint damage in rheumatoid arthritis. Arthritis Rheum. **58**:3388–3393.
66. **Walter RB, Bachli EB, Schaer DJ, Ruegg R, Schoedon G.** 2003. Expression of the hemoglobin scavenger receptor (CD163/HbSR) as immunophenotypic marker of monocytic lineage in acute myeloid leukemia. Blood **101**:3755–3756.
67. **Weaver LK, et al.** 2006. Pivotal advance: activation of cell surface Toll-like receptors causes shedding of the hemoglobin scavenger receptor CD163. J. Leukoc. Biol. **80**:26–35.
68. **Winterbourn CC.** 1985. Handbook of methods for oxygen radical research, vol 1. CRC Press, Boca Raton, FL.
69. **Yamada N, et al.** 2000. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. Am. J. Hum. Genet. **66**:187–195.
70. **Yano A, Yamamoto Y, Miyaishi S, Ishizu H.** 1998. Haptoglobin genotyping by allele-specific polymerase chain reaction amplification. Acta Med. Okayama **52**:173–181.

5.3 MANUSCRITO III

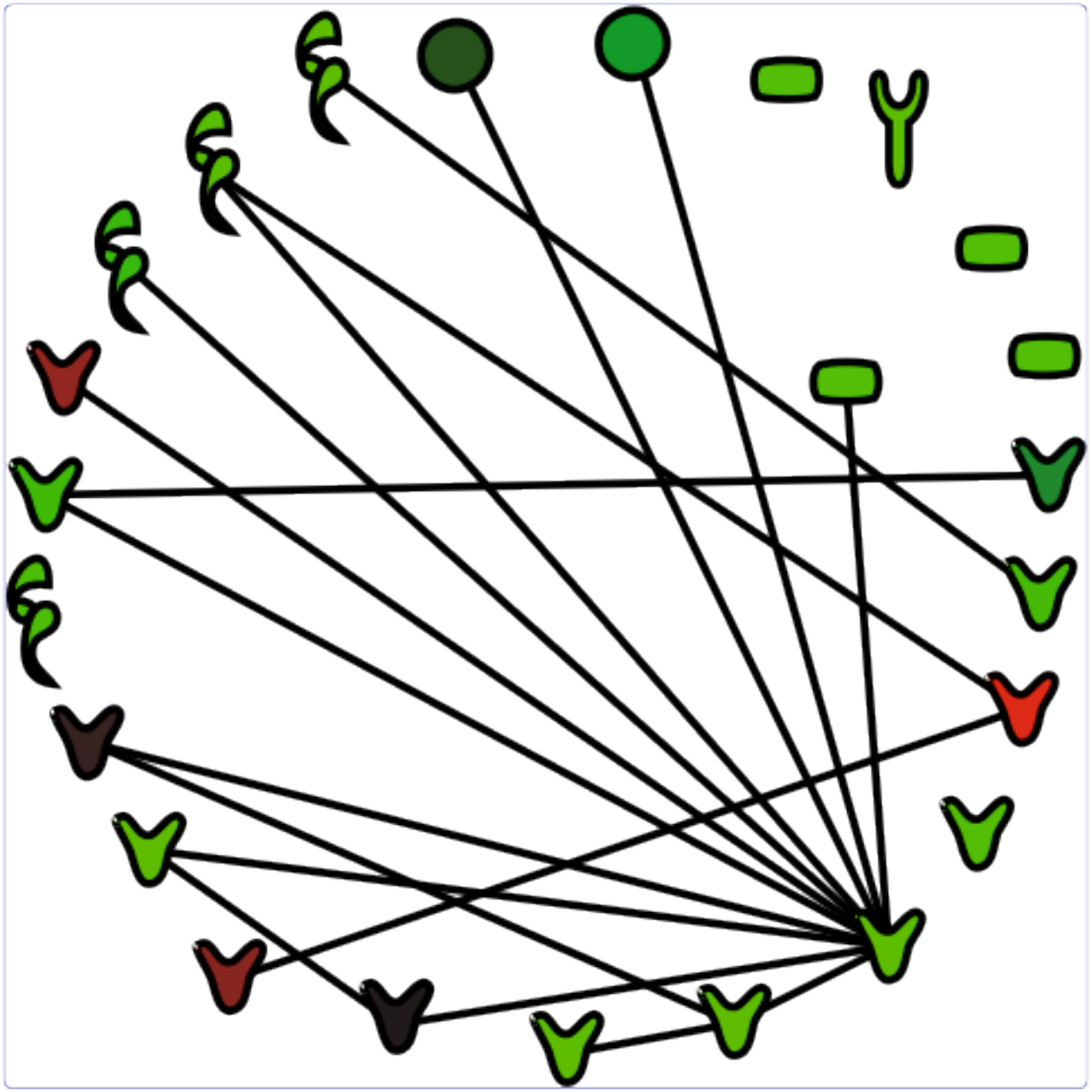
Networking the host immune response in *Plasmodium vivax* malaria.

Este trabalho investiga a interação entre biomarcadores plasmáticos relacionados a respote imune e inflamatória por uma abordagem analítica de redes, tentando buscar padrões associados com a infecção pelo *P. vivax*.

Resumo dos resultados: Indivíduos não-infectados demonstraram diversas interações estatisticamente significante nas redes, incluindo associações entre os níveis de IL-10 e IL-4 com a quimiocina motivo C-X-C ligante (CXCL)9. Participantes com malária assintomática tiveram várias correlações significativas envolvendo a IL-4. Indivíduos com malária moderada ou malária grave sem mortalidade, por sua vez, apresentaram uma perda notável de interações nas redes e o TNF foi a molécula com mais associações significativas com os outros parâmetros. Por fim, os casos de malária grave com óbito foram associados com correlações sisgnificativas entre o TNF, alanina aminotransferase (ALT), HO-1 e SOD-1.

Este trabalho foi publicado no periódico internacional *Malaria Journal.*

MALARIA JOURNAL



Networking the host immune response in *Plasmodium vivax* malaria

Mendonça *et al.*

BioMed Central

Mendonça *et al. Malaria Journal* 2013, **12**:69
http://www.malariajournal.com/content/12/1/69

MALARIA JOURNAL

RESEARCH Open Access

Networking the host immune response in *Plasmodium vivax* malaria

Vitor RR Mendonça[1,2], Artur TL Queiroz[1], Fabrício M Lopes[3,4], Bruno B Andrade[5*†] and Manoel Barral-Netto[1,2,6†]

Abstract

Background: *Plasmodium vivax* malaria clinical outcomes are a consequence of the interaction of multiple parasite, environmental and host factors. The host molecular and genetic determinants driving susceptibility to disease severity in this infection are largely unknown. Here, a network analysis of large-scale data from a significant number of individuals with different clinical presentations of *P. vivax* malaria was performed in an attempt to identify patterns of association between various candidate biomarkers and the clinical outcomes.

Methods: A retrospective analysis of 530 individuals from the Brazilian Amazon, including *P. vivax*-infected individuals who developed different clinical outcomes (148 asymptomatic malaria, 187 symptomatic malaria, 13 severe non-lethal malaria, and six severe lethal malaria) as well as 176 non-infected controls, was performed. Plasma levels of liver transaminases, bilirubins, creatinine, fibrinogen, C-reactive protein, superoxide dismutase (SOD)-1, haem oxygenase (HO)-1 and a panel composed by multiple cytokines and chemokines were measured and compared between the different clinical groups using network analysis.

Results: Non-infected individuals displayed several statistically significant interactions in the networks, including associations between the levels of IL-10 and IL-4 with the chemokine CXCL9. Individuals with asymptomatic malaria displayed multiple significant interactions involving IL-4. Subjects with mild or severe non-lethal malaria displayed substantial loss of interactions in the networks and TNF had significant associations more frequently with other parameters. Cases of lethal *P. vivax* malaria infection were associated with significant interactions between TNF ALT, HO-1 and SOD-1.

Conclusions: The findings imply that clinical immunity to *P. vivax* malaria is associated with multiple significant interactions in the network, mostly involving IL-4, while lethality is linked to a systematic reduction of complexity of these interactions and to an increase in connections between markers linked to haemolysis-induced damage.

Keywords: Malaria, *Plasmodium vivax*, Biomarkers, Network analysis

Background

The vast majority of human diseases do not result from single molecular changes, and malaria is certainly included in this group. Besides factors related to the *Plasmodium* parasite itself, malaria is a consequence of multiple host molecular and genetic alterations compounded by environmental factors [1]. Most of the studies in malaria immunopathogenesis are focused on the identification of one or a small group of candidate molecules related to disease severity. The cytokine balance seems to influence malaria outcome, with TNF, IFN-γ and IL-10 emerging as key players in both experimental models and in human observational studies [2-6]. Recently, host factors associated with oxidative stress, such as the enzymes superoxide dismutase 1 (SOD-1) and haem oxygenase (HO)-1, as well as molecules that are linked to metabolic adaptation to iron overload during malaria-triggered haemolysis, have been identified as very potent biomarkers to distinguish individuals developing more severe forms of vivax malaria [7-9]. However, the patterns of interaction between these multiple factors within the clinical spectrum of human malaria are largely unexplored. The situation is

* Correspondence: andradebbd@niaid.nih.gov
†Equal contributors
[5]Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20893, USA
Full list of author information is available at the end of the article

BioMed Central

© 2013 Mendonça et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

even worse concerning the infection caused by *Plasmodium vivax*, which has been relatively neglected for many years and recently re-emerged as a potentially lethal disease [6,10-13].

The use of a comprehensive approach to exploring the relationship between groups of molecules likely to be related to the severity of the disease manifestations may contribute to a better understanding of the patterns of susceptibility to disease severity or disease tolerance. In this study, network analysis of a large dataset from a significant number of individuals presenting with different clinical manifestations of vivax malaria was performed in an attempt to identify patterns of association between various candidate biomarkers and the clinical outcomes. The findings from this exploratory study demonstrate a new level of complexity connecting several mediators involved in the immunopathogenesis of vivax malaria.

Methods

Study design and participants

The present study is a retrospective analysis of a databank containing clinical, epidemiological and immunological data from 530 individuals from the Brazilian Amazon (Rondônia, Brazil) recruited between 2006 and 2007, as part of a project aimed at describing determinants of susceptibility to vivax malaria that was finalized on 2010. In this project, both active and passive malaria case detection were performed. These included home visits in areas of high transmission (active case detection), and study of individuals seeking care at the diagnostic centres of Brazilian National Foundation of Health (FUNASA) or in a municipal hospital in Buritis, Rondônia, Brazil (passive case detection). Individuals of both sexes, ranging in age from five to 70 years, who had resided in the endemic area for more than six months, were invited to participate. The details of the recruitment, diagnosis approach and clinical definitions of asymptomatic infection or severe malaria caused by *P. vivax* used in the project were published previously [6-9,14-17]. The exclusion criteria were as follows: *Plasmodium falciparum* infection documented by both microscopy and nested polymerase chain reaction (PCR), documented viral hepatitis, chronic alcoholism, human immunodeficiency virus type 1 infection, yellow fever, leptospirosis, cancer and chronic degenerative diseases, sickle cell trait and the use of hepatotoxic or immunosuppressant drugs. The malaria diagnosis was made in a reference centre from the National Foundation of Health in the endemic area and confirmed with nested PCR as previously described [14,15,17]. Individuals without symptoms were actively recruited in their residencies by active search, mostly in remote riverine communities and had thick blood smear samples and a small aliquot of blood in EDTA tubes collected for diagnostic screening by nested PCR. The diagnosis in those individuals was performed after the clinical visit and if a positive test for malaria was found, a second visit within 30 days was performed in order to search for appearance of malaria symptoms. A new sample collection was made and a second round of diagnostic tests was performed. Subjects that remained with positive nested PCR for *Plasmodium* within this period of 30 days and presented no malaria-related symptoms, such as fever (axillary temperature >37.8°C), chills, sweats, myalgia, arthralgia, strong headaches, nausea, vomiting, jaundice, and severe asthenia during this period were considered as asymptomatic malaria cases. During the first clinical visit (previously to the results of the diagnostic tests) all the asymptomatic individuals received counselling and were oriented to seek for health care in a malaria reference centre in case symptoms appeared during the period between the 2 clinical visits. After the second clinical visit, all the patients that had positive malaria screening with microscopy and/or nested PCR were treated following the treatment guidelines of the Brazilian Ministry of Health. The plasma samples used to assess the biomarkers were collected during the second clinical visit, before the initiation of anti-malarial drugs. Symptomatic individuals were promptly treated. Data from a total of 530 individuals were analysed, and the individuals were stratified into the following categories: non-infected (n=176), asymptomatic vivax malaria (n=148), symptomatic vivax malaria (n=187), severe non-lethal vivax malaria (n=13), and severe vivax malaria associated with mortality (n=6). The plasma samples obtained from patients with severe forms of malaria, including the cases that eventually died during the follow up were collected at the hospital admission and the procedures are described elsewhere [6]. Demographic characteristics of the study participants are shown in the Table 1.

Ethics statement

Written informed consent was obtained from all participants or their legally responsible guardians, and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The project was approved by the institutional review board of the Faculdade de Medicina, Faculdade São Lucas, Rondônia, Brazil, where the study was performed.

Laboratory measurements

Several mediators were selected based on the assessment of the overall inflammatory status and immune responses in malaria. All the biomarkers that were measured in all the patients, and were contained in the databank, were included in the analysis and no pre-selection was done. Plasma measurements of aspartate amino-transferase (AST), alanine amino-transaminase (ALT), total bilirubin, direct bilirubin, creatinine, fibrinogen and C-reactive

Table 1 Demographic characteristics of the participants

	Endemic controls	Asymptomatic malaria	Symptomatic malaria	Severe malaria		P-value
				Survivors	Deaths	
	n=176	n=148	n=187	n=13	n=6	
Male (%)	72 (40.9)	70 (47.3)	93 (49.7)	7 (53.8)	3 (50.0)	0.504
Age - years						<0.001
Median	32	40	33	22	27	
IQR	24-45	32-49	27-42	16-30	13-44	
Years residing in the area						<0.001
Median	12.6	11.8	7.6	2.6	3	
IQR	3.2-14.8	3.5-16.4	0.6-10.1	0.5-4.8	0.3-5.2	
Parasites/μL						<0.001
<500	176	145	49	0	0	
	(100%)	(98.0%)	(26.2%)			
500-5,000	0	3(2.0%)	84	4	1	
			(44.9%)	(30.8%)	(16.7%)	
5,001-50,000	0	0	50	6	3	
			(26.7%)	(46.1%)	(50%)	
>50,000	0	0	4	3	2	
			(2.1%)	(23.1%)	(33.3%)	

IQR: interquartile range.

Table 2 Distribution of cytokines levels in the study subjects stratified by *Plasmodium vivax* malaria clinical outcome

Biomarker	Endemic controls	Asymptomatic malaria	Symptomatic malaria	Severe malaria: Survivors	Severe malaria: Deaths	P value 1	P value 2
	n=176	n=148	n=187	n=13	n=6		
IL-1β	5.7	4.0	11.4	7.6	15.54	<0.001	<0.001
pg/mL	(3.5-17)	(2.7-7.8)	(6.2-25.5)	(6.6-23.8)	(4.8-29.3)		
IL-4	23.4	22.1	29.89	30.1	36.4	<0.001	0.008
pg/mL	(12.3-40)	(12-34.4)	(16.8-102)	(18.5-41)	(18-115)		
IL-6	8.4	10.3	69.2	78.5	101.2	<0.001	<0.001
pg/mL	(5.2-20)	(1.5-21.4)	(23.9-105.5)	(56-105)	(41-140)		
IL-8	6.3	3.6	26.0	12.4	66.8	<0.001	<0.001
pg/mL	(4.7-11)	(2.3-9.2)	(5.9-102.5)	(6.1-66.7)	(15-211)		
IL-10	12.0	62.0	125.0	140.2	110.5	<0.001	0.349
pg/mL	(7-20.4)	(11.3-89.6)	(65.3-455.1)	(85-550)	(79-134)		
IL-12p70	7.7	13.5	20.7	12.7	10.0	<0.001	0.520
pg/mL	(4.9-16)	(7.7-18.3)	(12.4-30.5)	(5.1-20.9)	(5-15)		
IFN-γ	32.1	54.3	103.4	212.5	181.6	<0.001	<0.001
pg/mL	(11-62)	(23.6-142.0)	(42.0-324.0)	(80-465)	(65-357)		
TNF-α	0	2.4	38.5	57.2	31.95	<0.001	<0.001
pg/mL	(0-10.5)	(0-10.3)	(18.1-80.5)	(32.5-84)	(19-76.8)		
TGF-β	23.8	89.8	111.1	48.6	40.2	<0.001	<0.001
pg/mL	(1.3-31)	(39.4-193.8)	(84.5-364.7)	(32-58.6)	(26-44.5)		

Note: Data represent median values and interquartile ranges. P value 1 was obtained using Kruskal-Wallis test, whereas P value 2 was calculated using Linear trend post test.
IQR: interquartile range.

protein (CRP) were made at the clinical laboratory of Faculdade São Lucas and at the Pharmacy School (Federal University of Bahia, Brazil). The cytokines IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, TNF and the chemokines CCL2 (MCP-1), CCL5 (RANTES), CXCL9 (MIG) and CXCL10 (IP-10) were measured using the cytometric bead array (CBA) (BD Biosciences Pharmingen, San Diego, CA, USA). The flow cytometric assay was performed and analysed by a single operator, and standard curves were derived from cytokine standards. The experiments were performed according to the manufacturers' instructions. ELISA kits were used to measure the soluble TNF receptor I (sTNF-RI; R&D Systems, Minneapolis, MN, USA), transforming growth factor (TGF)-β (R&R Systems), SOD-1 (Calbiochem, San Diego, CA, USA) and HO-1 (Assay Designs, Ann Arbor, MI, USA) according to the manufacturers' protocols. In order to compare the distribution of the biomarkers according to the clinical groups, the laboratory parameters were categorized in cytokines (Table 2), surrogates of inflammatory damage (Table 3) and chemokines and other proteins (Table 4).

Network analysis

The inferential network was generated from the values of each mediator measured in the plasma samples, in which it was observed that patterns of the concentrations and clinical classification of the subjects were based on the disease outcomes. The systemic levels of each mediator were input in the DimReduction software [18]. As a result, the DimReduction software selected the patterns of distribution of the mediators associated with each clinical group. Moreover, the same software was applied to identify links of interaction between the mediators. Following this approach, each mediator is selected as a target and the DimReduction software performs a search within the other mediators for those that are associated with the target in terms of entropy. As a result, the features related to the selected target are linked. This process is repeated by considering each mediator at a time and the result is the inferred network among the input values. The DimReduction default parameters values were kept fixed during all the experiments.

In order to analyse the structure of the biomarker networks, the network density was adopted, which is, in the context of this work, the ratio of the number of edges inferred in the network over total number of possible edges between all pairs of nodes. The density measure is defined as follows: density = L/(N (N-1)/2), in which L is the number of observed edges and N is the total number of the nodes in the network. The density is normalized, ranging between 0 (no edges in the network) and 1 (all possible edges presents).

Table 3 Assessment of inflammatory damage in the study subjects stratified by *Plasmodium vivax* malaria clinical outcome

Biomarker	Endemic controls	Asymptomatic malaria	Symptomatic malaria	Severe malaria: Survivors	Severe malaria: Deaths	P value 1	P value 2
	n=176	n=148	n=187	n=13	n=6		
Total bilirubin mg/dL	0.7	0.8	1.2	1.8	2.1	<0.001	<0.001
	(0.5-1.1)	(0.5-1.2)	(0.8-1.9)	(1.5-2.5)	(1.2-3.2)		
Direct bilirubin mg/dL	0.3	0.4	0.4	0.6	1.1	<0.001	<0.001
	(0.2-0.4)	(0.3-0.8)	(0.3-0.8)	(0.4-1.3)	(0.3-1.7)		
Indirect bilirubin mg/dL	0.4	0.3	0.7	1.1	1.2	<0.001	<0.001
	(0.3-0.6)	(0.2-0.4)	(0.4-1.2)	(0.8-1.3)	(0.9-1.3)		
AST U/L	43.3	56.4	167	201.0	268.4	<0.001	<0.001
	(34-56.3)	(35.5-87.5)	(81.5-506)	(87-302)	(160-340)		
ALT U/L	40.9	44.9	180	201.1	278.8	<0.001	<0.001
	(33.5-54)	(32-69.4)	(123--438)	(190-304)	(175-342.9)		
Creatinine mg/dL	1.2	1.2	1.3	1.7	2.4	<0.001	<0.001
	(1.1-1.3)	(1.0-1.3)	(1.2-1.4)	(1.3-2.5)	(1.9-2.5)		
CRP mg/L	5.2	7.9	15.5	13.2	34.4	<0.001	<0.001
	(3.8-9.7)	(4.8-12.3)	(8.2-32.8)	(6.7-47.5)	(16.4-50.7)		
Fibrinogen mg/dL	234.0	302.3	374.5	415.5	437.7	<0.001	<0.001
	(198-305)	(210.4-377.5)	(234-485.6)	(374-498)	(348.5-530.8)		

Note: Data represent median values and interquartile ranges. P value 1 was obtained using Kruskal-Wallis test, whereas P value 2 was calculated using Linear trend post test.
CRP: C-reactive protein.

Table 4 Distribution of chemokines and other proteins levels in the study subjects stratified by *Plasmodium vivax* malaria clinical outcome

Biomaker	Endemic controls	Asymptomatic malaria	Symptomatic malaria	Severe malaria: Survivors	Severe malaria: Deaths	P value 1	P value 2
	n=176	n=148	n=187	n=13	n=6		
CCL2	86.0	85.2	65.8	64.7	87.9	0.117	0.207
ng/mL	(21-176)	(18-161.6)	(23.0-145.6)	(43-127)	(34.7-139)		
CCL5	27.0	24.4	25. 3	26.1	36.7	0.007	0.156
µg/mL	(15-45.7)	(13.4-38)	(15.8-70.2)	(20-38.7)	(25.4-83.2)		
CXCL9	0.3	0.5	2.2	3.5	4.6	<0.001	<0.001
ng/mL	(0.2-0.5)	(0.3-0.8)	(0.4-9.4)	(0. 6-12.5)	(0.8-9.9)		
CXCL10	88.0	19.4	77.4	117.7	183.3	<0.001	<0.001
pg/mL	(25-198)	(10.2-25.0)	(25.3-360.7)	(28-564)	(30.0-395.5)		
sTNF-RI	0.2	0.5	0.6	0.8	2.2	<0.001	<0.001
ng/mL	(0.1-0.4)	(0.4-0.6)	(0.4-0.7)	(0.7-0.9)	(1.7-3.1)		
SOD-1	4.2	6.0	26.0	72.6	82.4	<0.001	<0.001
ng/mL	(2.6-6.8)	(3.6-10)	(18.8-34.0)	(71-80.4)	(76.6-100.5)		
HO-1	29.1	29.5	35.5	42.9	49.2	<0.001	<0.001
ng/mL	(25.8-32)	(26.3-35.2)	(29.7-44.8)	(38.5-45)	(48.4-57.3)		

Note: Data represent median values and interquartile ranges. P value 1 was obtained using Kruskal-Wallis test, whereas P value 2 was calculated using Linear trend post test.

Statistical analysis

In the exploratory analysis of the data, frequency tables were constructed and the Chi-square test was applied to evaluate the association between qualitative variables. The quantitative variables were tested for Gaussian distribution within the total sample using D'Agostino and Pearson omnibus normality test. The variables with normal distribution were compared between the groups by one-way ANOVA with linear trend or Bonferroni's multiple comparisons post-test. Further analysis were based on non-parametric tests only, considering the small number of individuals located in the two groups with severe vivax malaria. In this context, Kruskal Wallis test was used to assess the differences between the clinical groups and Mann-Kendall test was used to estimate liner trends. Only results from the non-parametric analyses are shown. Correlations between parasitaemia and the biomarkers from the networks were assessed using the Spearman rank test. For each analysis, $P<0.05$ was considered statistically significant. The graphics for the network analysis were customized using the Ingenuity Systems Pathway Analysis software (Ingenuity Systems, Redwood City, CA, USA). The statistical analyses were performed using the programs GraphPad Prism 5.0 (GraphPad Software Inc, USA), STATA 9.0 (StataCorp, TX, USA), and JMP 9.0 (SAS, Cary, NC, USA).

Results

The plasma concentrations of most mediators measured were statistically different among the clinical groups, and many markers displayed linear trend to increase or decrease according to the degree of disease severity, as detailed in the Tables 2, 3 and 4. In the network analysis, densities of biomarker networks were observed, ranging between 0.021 and 0.032 in the different groups (Figure 1 and Figure 2A). The groups of non-infected individuals and those with asymptomatic malaria presented higher connectivity among the molecules, 0.030 and 0.032 respectively, compared to the groups of individuals with symptomatic infection (symptomatic malaria: 0.023; non-lethal severe disease: 0.021; and lethal malaria: 0.027) (Figure 2A).

In the group of non-infected individuals, CCL5 and CXCL9 had several significant interactions, and the latter was linked to the regulatory cytokines IL-10 and IL-4. In addition, CRP, ALT and IL-10 also presented relatively high connectivity, arguing that a fine tune of interactions involving these markers may influence the resistance against *P. vivax* infection. In those individuals in which infection did not lead to the onset of symptoms, the number of significant interactions was similar to the group of non-infected people (19 significant hits in the group of uninfected individuals and 18 hits in asymptomatic malaria), but the number of associations involving mainly IL-4 and to a lesser extent IL-1β was

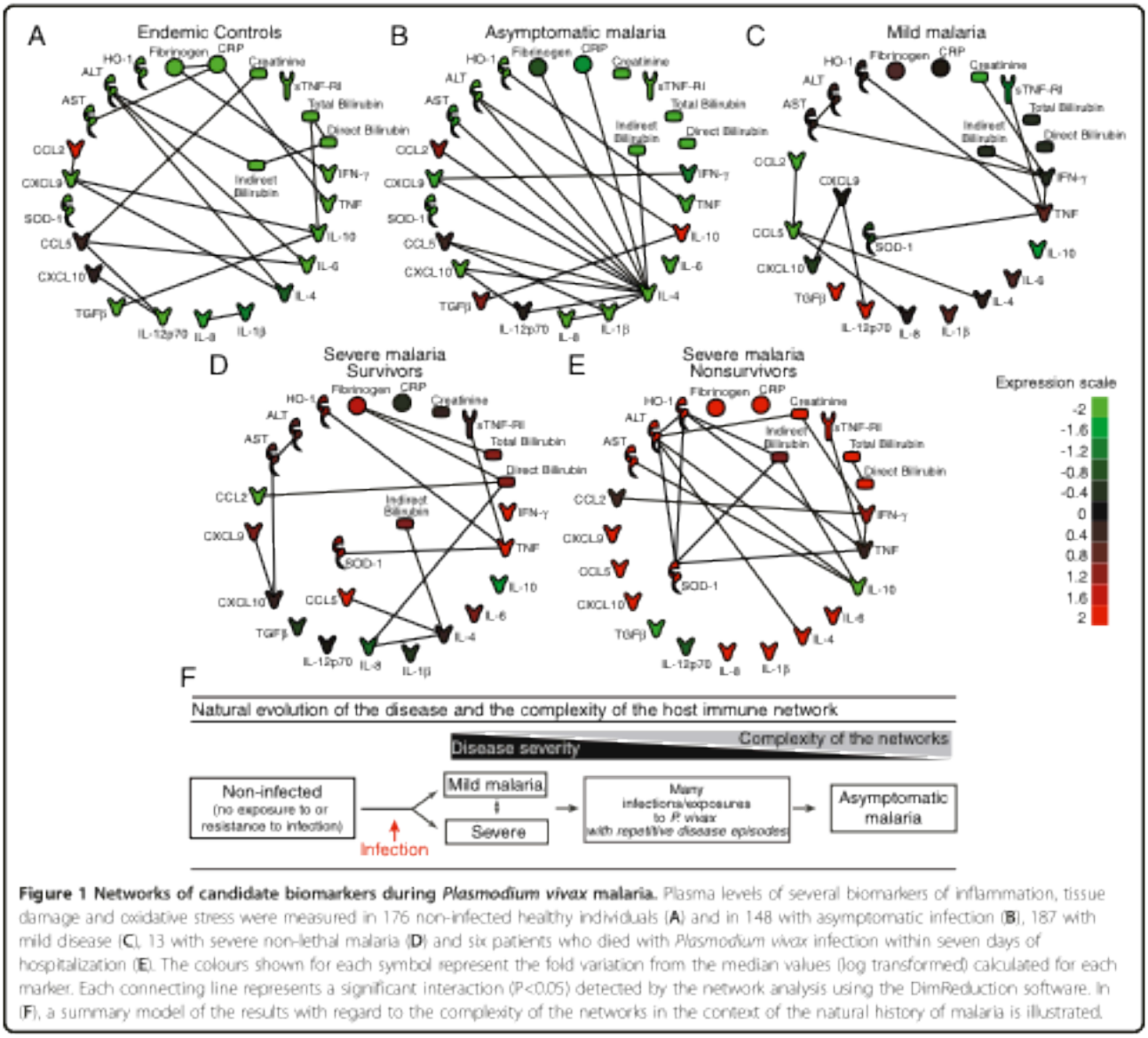


Figure 1 Networks of candidate biomarkers during *Plasmodium vivax* malaria. Plasma levels of several biomarkers of inflammation, tissue damage and oxidative stress were measured in 176 non-infected healthy individuals (**A**) and in 148 with asymptomatic infection (**B**), 187 with mild disease (**C**), 13 with severe non-lethal malaria (**D**) and six patients who died with *Plasmodium vivax* infection within seven days of hospitalization (**E**). The colours shown for each symbol represent the fold variation from the median values (log transformed) calculated for each marker. Each connecting line represents a significant interaction ($P<0.05$) detected by the network analysis using the DimReduction software. In (**F**), a summary model of the results with regard to the complexity of the networks in the context of the natural history of malaria is illustrated.

greatly increased in these individuals, leading to a totally different pattern of connectivity in the network (Figure 1).

In contrast, the patterns of connections between the mediators seen in the groups of individuals with symptomatic malaria were dramatically diverse than in the groups without the disease. The group of subjects with mild malaria displayed a substantial loss of the number of significant interactions in the network and the pro-inflammatory cytokines IFN-γ, TNF and CCL5 had the highest number of connections (Figure 1). IFN-γ was associated with indirect bilirubin, creatinine, AST and TNF, arguing that this cytokine could be driving inflammation (Figure 1). A number of other significant connections were detected between pro-inflammatory mediators, such as IL-12 and CXCL9. TNF-α was associated with IFN-γ, sTNF-RI, SOD-1 and HO-1, implying a potential interplay between those mediators in the pathogenesis of malaria-related symptoms. Intriguingly, the number of significant interactions and the overall complexity of networks were systematically reduced in the group of patients with severe forms of malaria (Figure 1). In the group of individuals who survived severe malaria, TNF, IL-4 and direct bilirubin seemed to have a distinguished pattern of connections, albeit, no clear clusters were evident and there was overall low connectivity between the markers studied. Moreover, in the group of individuals that succumbed to infection and died, TNF, ALT, HO-1, SOD-1, IL-10, IFN-γ and indirect bilirubin displayed more connectivity with other biomarkers, which caused a small increase in the complexity of the network when compared to the group of individuals with non-lethal severe malaria (Figure 1).

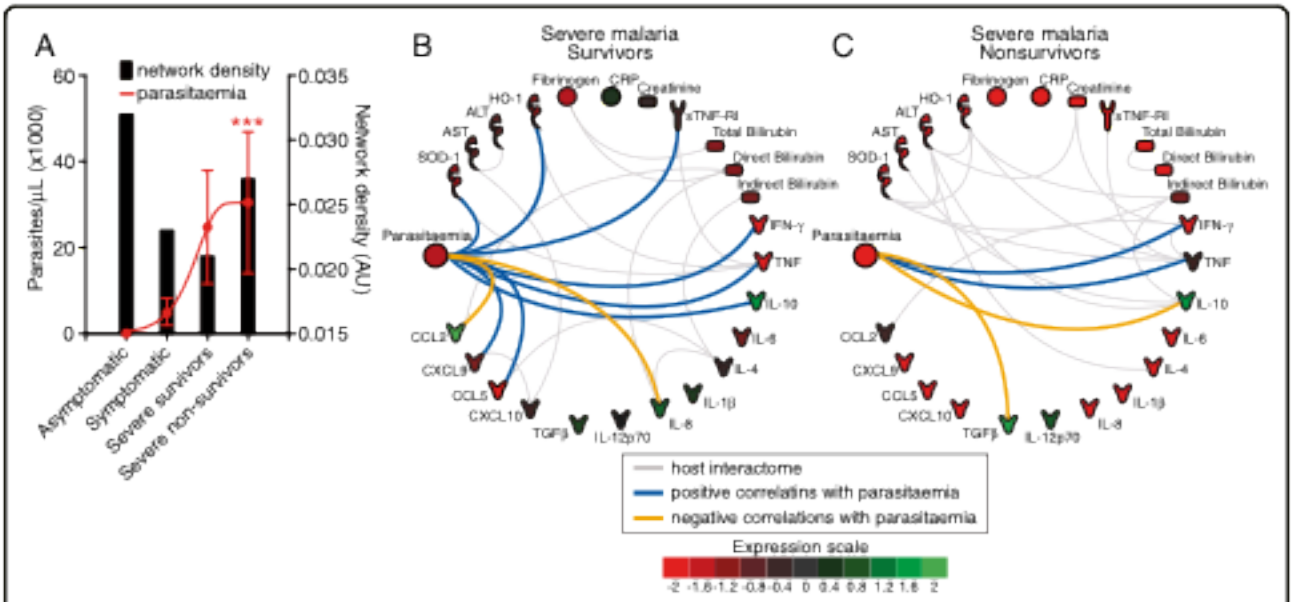


Figure 2 Associations between *Plasmodium vivax* parasitaemia and host biomarkers. (**A**) The distribution of *P. vivax* parasitaemia in different clinical groups is shown in red symbols (geometric means and 95% confidence intervals) whereas the values for network densities are shown as black bars. The variation of parasitaemia according to the malaria clinical severity was assessed using the Mann-Kendall test for linear trend (*** P<0.001). Statistically significant correlations between parasitaemia and different biomarkers are shown in (**B**) for the group of individuals with non-lethal severe malaria and in (**C**) for the group of patients that died during hospitalization. The correlations were assessed using the Spearman rank test. The correlations involving parasitaemia shown in (**B**) and (**C**) are plotted on top of the host interactome.

Because *P. vivax* parasite counts in the blood displayed a strong linear trend to increase according to the disease clinical severity in the study population (Table 1 and Figure 2A), the potential associations between parasitaemia levels and the pattern of expression of the different biomarkers from the networks were investigated in the groups of patients with elevated morbidity. In the group of individuals presenting with severe non-lethal malaria, parasitaemia displayed significant positive associations with TNF, sTNF-RI, IFN-γ, IL-10, CXCL9, CXCL10, SOD-1 and HO-1, while negatively correlated with the chemokines IL-8 and CCL2 (Figure 2B). Interestingly, the number of significant associations involving parasitaemia was greatly reduced in the group of patients that died with severe vivax malaria (Figure 2C). The positive correlations involving TNF and IFN-γ persisted in this group of highly susceptible patients who died, while the regulatory cytokines IL-10 and TGF-β were negatively correlated with parasitaemia (Figure 2C).

Discussion

The present study evaluated for the first time the density and complexity of the network of interactions between several markers strongly associated with host immune responses in their relationship with markers of tissue injury against *P. vivax* malaria in the context of the disease clinical outcomes. The results presented herein demonstrate that non-infected endemic controls exhibit a rich and dense network of interactions among immune-related markers and those involved in pathology. Such network is drastically deranged in symptomatic individuals but it is reconstituted in asymptomatic individuals. In this context it is stressed that asymptomatic infection is a state achieved by long-time residents in endemic areas and strongly related to several previous episodes of symptomatic malaria [6] being thus related to the development of a partial resistance to infection and/or tolerance to disease [19]. As a consequence, the onset of symptoms is prevented by limiting parasite burden and controlling inflammation. Such asymptomatic carriers develop just enough immunity to protect them from malarial illness, but not from malarial infection [1]. In the present study, asymptomatic carriers displayed the highest density values in the networks interactions, which argue that there may exist a complex regulatory mechanism behind the susceptibility to infection and tolerance and/or resistance against malaria. The network density values described in this study are similar to another protein-protein and metabolism-associated networks described previously in many other clinical contexts [20], which suggests that immune system may display similar homeostatic responses involving intrinsically complex regulatory pathways. Additional studies assessing network analysis of the immune responses in other infections and clinical contexts are needed to identify potential key common factors that could be targeted in a therapeutic approach.

The high connectivity exhibited by CXCL9 may be indicative of an important role for this chemokine in exposed but non-infected individuals since it correlates with protection against malaria in volunteers vaccinated by the circumsporozoite protein-based vaccine [21]. In addition, both IL-10 and IL-4 had significant connections with CXCL9, which also interacted with CCL2 and IFN-γ in those non-infected and with symptomless *P. vivax* infection, respectively. These interactions evidence a protective role of IL-4 and IL-10 through a modulatory effect on these pro-inflammatory mediators. Support for this modulatory hypothesis comes from the description that IL-10 and IL-4 can directly down regulate pro-inflammatory cytokines such as IL-6, TNF and IL-1β and prevent severe forms of malaria [22-26]. Studies with mouse macrophages *in vitro* argue that IL-4 suppresses the expression of CXCL9 and other IFN-γ inducible genes via STAT 6 signalling [27]. Although interesting, a role of a direct modulatory link between IL-10, IL-4 and CCL2 and/or CXCL9 and pro-inflammatory status of *Plasmodium* infected individuals is yet unexplored mechanistically and deserves further investigation.

Extensive research has been done in the identification of factors associated with severe forms of malaria, with significant contributions to the understanding of the disease pathogenesis. In the present study and not surprisingly, IFN-γ and TNF and the chemokine CCL5 were demonstrated to be crucial biomarkers in the network profile of those individuals with mild vivax malaria. CCL5 is a chemokine involved in the generation of inflammatory cellular infiltrates and its low levels are linked to cerebral malaria and severe malarial anaemia, maybe as consequence of thrombocytopaenia and the influence of haemozoin production [28-30]. The connectivity between IL-12 and CXCL9 seen in the networks from mildly symptomatic patients is also observed in the context of cancer therapy, in which administration of IL-12 increases the expression of CXCL9 in peripheral blood mononuclear cells as a response against the tumour [31,32]. Interactions involving CXCL9 had an intriguing pattern between the study groups. This chemokine was more associated with regulatory cytokines in the group of non-infected controls, which may suggest a potential influence over the resistance to infection. On the other side, CXCL9 was linked to pro-inflammatory IL-12 within symptomatic subjects, which may be associated with the onset of clinical disease.

While networking the immune response in lethal cases of severe *P. vivax* malaria, it was found an important role for HO-1, an enzyme primarily responsible for the host detoxification from harmful free haem and which produces carbon monoxide (CO), free iron and biliverdin. Higher HO-1 levels were described in patients with symptomatic vivax malaria and this enzyme may contribute (both very high and low levels) to disease severity [8,33]. Lower expression of HO-1 may result in greater availability of free haem resulting in severe malaria, while high expression of HO-1 could lead to increased synthesis of free iron, which also can be harmful as can promote oxidative damage [33]. Furthermore, SOD-1, previously associated with *P. vivax* malaria [7], displayed connectivity only in the groups of patients with symptomatic disease, especially in those individuals who died upon infection. Accumulation of SOD-1 without a compensatory elevation of catalase of glutathione enzymes could lead to accumulation of H_2O_2 and the production of more free radicals by Fenton's reaction [34]. Indeed, it has been described that both glutathione and catalase might be reduced during severe malaria [35] and high levels of SOD-1 could lead to the exacerbation of the oxidative stress and inflammation, aggravating the outcome of the disease. In addition, the release of haem during malaria erythrocytic cycle reduces the production of anti-inflammatory prostaglandin E_2 and TGF-β from mononuclear cells via SOD-1 [16]. Thus, SOD-1 connectivity seen herein in severe malaria patients seems to be a response against the high production of deleterious haem during the robust intravascular haemolysis observed in severe malaria.

Furthermore, TNF, IFN-γ and IL-10 also had important interactions in individuals who succumbed to malaria infection. IL-10 seems to modulate Th1-type responses to *Plasmodium* antigens by downregulating TNF, and IFN-γ levels by dampening the release of IL-12p70, thus resulting in an impaired immune response that may lead to lethality [5,36]. However, it has also been described that in cases of uncontrolled inflammation with high production of pro-inflammatory mediators may be associated with exhaustion of the protective regulatory responses [6]. In response to malaria infection, the patterns of expression of multiple cytokines and the relative balance and interactions between these factors may be capable of mediating protective immunity or disease severity depending of the context [37]. These findings infer that fine-tuning between all the mediators is extremely important to prevent malaria lethality.

A major observation from the analysis of the groups of patients with mild or severe malaria was that the overall complexity of the networks by means of a number of significant interactions was reduced while compared to what was seen in healthy subjects. Intriguingly, in the group of individuals with asymptomatic malaria, the complexity of the network was restored and several significant interactions were detected, but with a clear central role for IL-4. The potential anti-inflammatory effect of IL-4 in the immune response during malaria is not yet fully understood [38]. IL-4 seems to assist in antisporozoite immunity and studies in mice infected

with *Plasmodium yoelii* suggest that IL-4 is also required for the development of CD8+ T lymphocytes [39] and for the development of a memory response against liver stage parasite [40]. Furthermore, IL-4 interferes with the maturation of Th1 cells and can reduce the production of IFN-γ in some experimental settings [41]. The high number of interactions between IL-4 and others biomarkers in asymptomatic individuals highlights a possible importance of this molecule in modulating the disease tolerance of individuals constantly exposed to *P. vivax* and further investigations should address in detail the importance of IL-4 in symptomless malaria. A summary of the results with regard to the complexity of the network connectivity in the context of the natural evolution of vivax malaria is shown in Figure 1F.

In the groups of patients with severe malaria, *P. vivax* parasitaemia displayed several positive interactions with previously described inflammatory mediators that participate in the immunopathogenesis of malaria, such as TNF, IFN-γ and IL-10 [1,6,7] in addition to TGF-β. Surprisingly, liver transaminases, bilirubins, creatinine and CRP were not correlated with parasitaemia. The lack of direct interactions between parasitaemia and laboratory surrogates of inflammation-driven tissue injury and oxidative stress suggests that the parasite burden may influence more stringently the host immune response rather than induce lethal tissue damage itself. This idea, if validated in other studies, reinforces the argument that susceptibility of disease severity in vivax malaria might be a case of dysfunction of the host homeostatic system caused by an inflammatory imbalance driven by lack of resistance against *P. vivax*.

Conclusion

In summary, the systematic analysis of several mediators of inflammation measured simultaneously was able to characterize the overall pattern of immune response of patients with *P. vivax* malaria according to disease severity and clinical outcome. This approach also revealed other levels of complexity of the disease, involving intricate associations between unique markers, such as TNF, IFN-γ, IL-4, HO-1 and SOD-1. IL-4, despite being at low systemic levels, seems to be a central mediator with several significant interactions in individuals with asymptomatic malaria. In addition, connectivity involving CXCL9 and IFN-γ was more prevalent in the groups of individuals non-infected or with mild manifestations of the disease while associations involving SOD-1 and HO-1 were more evident in more severe and lethal cases. In severe malaria, parasitaemia seems to positively associate with pro-inflammatory mediators, and the simultaneous negative correlations involving the regulatory cytokines IL-10 and TGF-β argue that there is a deregulated balance of the host immune response that is skewed towards amplification of inflammation. Networking analysis may represent a potential tool to understand the interactions between several mediators in the context of malarial disease severity. The identification of critical factors driving malaria pathogenesis can guide future therapeutic approaches.

Abbreviations

(SOD)-1: Superoxide dismutase; (HO)-1: Haem oxygenase; FUNASA: Brazilian national foundation of health; PCR: Polymerase chain reaction; AST: Aspartate amino-transferase; ALT: Alanine amino-transaminase; CRP: C-reactive protein; CBA: Cytometric bead array; sTNF-RI: TNF receptor I; (TGF)-β: Transforming growth factor; CO: Carbon monoxide.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VRRM helped interpreting the data and to write the manuscript together with BBA and MB-N. AQ and FML performed the network analysis. BBA conceptualized the study, coordinated the clinical assessments and performed the laboratory experiments, analysed the data and wrote the first draft of the manuscript. MB-N supervised the clinical study and helped with data interpretation and the writing of the manuscript. All authors have read and approved the final version of the manuscript.

Acknowledgements

The authors thank David Kugler (NIAID, NIH, USA) for critical help with the data analysis and helpful discussions. A special thank to João Gambati (FUNASA, Brazil), Luis Marcelo Camargo (USP, Brazil) and the study team at FIOCRUZ-Bahia, Brazil, Daniela Andrade, Nívea Luz, Jorge Tolentino, Adorielze Leite, and Drs Aldina Barral and Jorge Clarêncio for help in logistics of the field study and experiments involving cytokine measurements. This work was supported by FINEP/FNDCT-CT Amazônia [field work; grant # 0104096605] and by FIOCRUZ. MB-N is a senior investigator from CNPq. VRRM is supported by CNPq. The work of BBA is supported by the intramural research programme of the National Institute of Allergy and Infectious Diseases, National Institute of Health. The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Author details

[1]Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brazil. [2]Faculdade de Medicina, Universidade Federal da Bahia, Salvador, Brazil. [3]Instituto de Matemática e Estatística, Universidade de São Paulo, São Paulo, Brazil. [4]Coordenação de Informática, Universidade Tecnológica Federal do Paraná, Comélio Procópio, Brazil. [5]Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20893, USA. [6]Instituto de Investigação em Imunologia, Instituto Nacional de Ciência e Tecnologia, São Paulo, Brazil.

Received: 20 January 2013 Accepted: 18 February 2013
Published: 21 February 2013

References

1. Andrade BB, Barral-Netto M: **Biomarkers for susceptibility to infection and disease severity in human malaria.** *Mem Inst Oswaldo Cruz* 2011, **106**:70–78.
2. Villegas-Mendez A, Greig R, Shaw TN, de Souza JB, Gwyer Findlay E, Stumhofer JS, Hafalla JCR, Blount DG, Hunter CA, Riley EM, Couper KN: **IFN-γ-Producing CD4+ T cells promote experimental cerebral malaria by modulating CD8+ T Cell accumulation within the brain.** *J Immunol* 2012, **189**:968–979.
3. Nilkura M, Kamiya S, Nakane A, Kita K, Kobayashi F: **IL-10 plays a crucial role for the protection of experimental cerebral malaria by co-infection with non-lethal malaria parasites.** *Int J Parasitol* 2010, **40**:101–108.
4. Seixas E, Oliveira P, Moura Nunes JF, Coutinho A: **An experimental model for fatal malaria due to TNF-alpha-dependent hepatic damage.** *Parasitology* 2008, **135**:683–690.

5. Ayimba E, Hegewald J, Ségbéna AY, Garnier RG, Lechner CJ, Agosssou A, Banla M, Soboslay PT: **Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe *Plasmodium falciparum* malaria.** *Clin Exp Immunol* 2011, **166**:218–226.
6. Andrade BB, Reis-Filho A, Souza-Neto SM, Clarêncio J, Camargo LMA, Barral A, Barral-Netto M: **Severe *Plasmodium vivax* malaria exhibits marked inflammatory imbalance.** *Malar J* 2010, **9**:13.
7. Andrade BB, Reis-Filho A, Souza-Neto SM, Raffaele-Netto I, Camargo LMA, Barral A, Barral-Netto M: **Plasma superoxide dismutase-1 as a surrogate marker of vivax malaria severity.** *PLoS Negl Trop Dis* 2010, **4**:e650.
8. Mendonça VRR, Luz NF, Santos NJG, Borges VM, Gonçalves MS, Andrade BB, Barral-Netto M: **Association between the haptoglobin and heme oxygenase 1 genetic profiles and soluble CD163 in susceptibility to and severity of human malaria.** *Infect Immun* 2012, **80**:1445–1454.
9. Gozzelino R, Andrade BB, Larsen R, Luz NF, Vanoaica L, Seixas E, Coutinho A, Cardoso S, Rebelo S, Poli M, Barral-Netto M, Darshan D, Kühn LC, Soares MP: **Metabolic adaptation to tissue iron overload confers tolerance to malaria.** *Cell Host Microbe* 2012, **12**:693–704.
10. Alexandre MA, Ferreira CO, Siqueira AM, Magalhães BL, Mourão MPG, Lacerda MV, GC AMd: **Severe *Plasmodium vivax* malaria, Brazilian Amazon.** *Emerg Infect Dis* 2010, **16**:1611–1614.
11. Lacerda MVG, Fragoso SCP, Alecrim MGC, Alexandre MAA, Magalhães BML, Siqueira AM, Ferreira LCL, Araújo JR, Mourão MPG, Ferrer M, Castillo P, Martin-Jaular L, Fernandez-Becerra C, Del Portillo H, Ordi J, Alonso PL, Bassat Q: **Postmortem characterization of patients with clinical diagnosis of *Plasmodium vivax* malaria: to what extent does this parasite kill?** *Clin Infect Dis* 2012, **55**:e67–74.
12. Mahgoub H, Gasim GI, Musa IR, Adam I: **Severe *Plasmodium vivax* malaria among Sudanese children at New Halfa Hospital.** *Eastern Sudan. Parasit Vectors* 2012, **5**:154.
13. Sharma R, Gohain S, Chandra J, Kumar V, Chopra A, Chatterjee S, Aneja S, Dutta AK: ***Plasmodium vivax* malaria admissions and risk of mortality in a tertiary-care children's hospital in North India.** *Paediatr Int Child Health* 2012, **32**:152–157.
14. Andrade BB, Rocha BC, Reis-Filho A, Camargo LMA, Tadei WP, Moreira LA, Barral A, Barral-Netto M: **Anti-*Anopheles darlingi* saliva antibodies as marker of *Plasmodium vivax* infection and clinical immunity in the Brazilian Amazon.** *Malar J* 2009, **8**:121.
15. Andrade BB, Reis-Filho A, Barros AM, Souza-Neto SM, Nogueira LL, Fukutani KF, Camargo EP, Camargo LMA, Barral A, Duarte A, Barral-Netto M: **Towards a precise test for malaria diagnosis in the Brazilian Amazon: comparison among field microscopy, a rapid diagnostic test, nested PCR, and a computational expert system based on artificial neural networks.** *Malar J* 2010, **9**:117.
16. Andrade BB, Araújo-Santos T, Luz NF, Khouri R, Bozza MT, Camargo LMA, Barral A, Borges VM, Barral-Netto M: **Heme impairs prostaglandin E2 and TGF-beta production by human mononuclear cells via Cu/Zn superoxide dismutase: insight into the pathogenesis of severe malaria.** *J Immunol* 2010, **185**:1196–204.
17. Andrade BB, Santos CJN, Camargo LM, Souza-Neto SM, Reis-Filho A, Clarêncio J, Mendonça VRR, Luz NF, Camargo EP, Barral A, Silva AAM, Barral-Netto M: **Hepatitis B infection is associated with asymptomatic malaria in the Brazilian Amazon.** *PLoS One* 2011, **6**:e19841.
18. Lopes FM, Martins DC, Cesar RM: **Feature selection environment for genomic applications.** *BMC Bioinformatics* 2008, **9**:451.
19. Medzhitov R, Schneider DS, Soares MP: **Disease tolerance as a defense strategy.** *Science* 2012, **335**:936–41.
20. Frankenstein Z, Alon U, Cohen IR: **The immune-body cytokine network defines a social architecture of cell interactions.** *Biol Direct* 2006, **1**:32.
21. Dunachie SJ, Berthoud T, Keating SM, Hill AVS, Fletcher HA: **MIG and the regulatory cytokines IL-10 and TGF-β1 correlate with malaria vaccine immunogenicity and efficacy.** *PLoS One* 2010, **5**:e12557.
22. Wilson NO, Bythwood T, Solomon W, Jolly P, Yatich N, Jiang Y, Shuaib F, Adjei AA, Anderson W, Stiles JK: **Elevated levels of IL-10 and G-CSF associated with asymptomatic malaria in pregnant women.** *Infect Dis Obstet Gynecol* 2010, **2010**. doi:10.1155/2010/317430. pii:317430.
23. Othoro C, Lal AA, Nahlen B, Koech D, Orago AS, Udhayakumar V: **A low interleukin-10 tumor necrosis factor-alpha ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya.** *J Infect Dis* 1999, **179**:279–82.
24. May J, Lell B, Luty AJ, Meyer CG, Kremsner PG: **Plasma interleukin-10: Tumor necrosis factor (TNF)-alpha ratio is associated with TNF promoter variants and predicts malarial complications.** *J Infect Dis* 2000, **182**:1570–3.
25. Kurtzhals JA, Adabayeri V, Goka BQ, Akanmori BD, Oliver-Commey JO, Nkrumah FK, Behr C, Hviid L: **Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria.** *Lancet* 1998, **351**:1768–72.
26. de Waal MR, Abrams J, Bennett B, Figdor CG, de Vries JE: **Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes.** *J Exp Med* 1991, **174**:1209–20.
27. Ohmori Y, Hamilton TA: **STAT6 is required for the anti-inflammatory activity of interleukin-4 in mouse peritoneal macrophages.** *J Biol Chem* 1998, **273**:29202–29209.
28. Were T, Hittner JB, Ouma C, Otieno RO, Orago ASS, Ong'echa JM, Vulule JM, Keller CC, Perkins DJ: **Suppression of RANTES in children with *Plasmodium falciparum* malaria.** *Haematologica* 2006, **91**:1396–1399.
29. John CC, Opika-Opoka R, Byarugaba J, Idro R, Boivin MJ: **Low levels of RANTES are associated with mortality in children with cerebral malaria.** *J Infect Dis* 2006, **194**:837–845.
30. Were T, Davenport GC, Yamo EO, Hittner JB, Awandare GA, Otieno MF, Ouma C, Orago ASS, Vulule JM, Ong'echa JM, Perkins DJ: **Naturally acquired hemozoin by monocytes promotes suppression of RANTES in children with malarial anemia through an IL-10-dependent mechanism.** *Microbes Infect* 2009, **11**:811–819.
31. Halicheur N, Escudier B, Dorval T, Negrier S, De Mulder PH, Dupuy JM, Novick D, Guillot T, Wolf S, Pouillart P, Fridman WH, Tartour E: **Cytokines and soluble cytokine receptor induction after IL-12 administration in cancer patients.** *Clin Exp Immunol* 2000, **119**:28–37.
32. Tannenbaum CS, Tubbs R, Armstrong D, Finke JH, Bukowski RM, Hamilton TA: **The CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor.** *J Immunol* 1998, **161**:927–932.
33. Takeda M, Kikuchi M, Ubalee R, Na-Bangchang K, Ruangweerayut R, Shibahara S, Imai S-I, Hirayama K: **Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to cerebral malaria in Myanmar.** *Jpn J Infect Dis* 2005, **58**:268–271.
34. Marikovsky M, Ziv V, Nevo N, Harris-Cerruti C, Mahler O: **Cu/Zn superoxide dismutase plays important role in immune response.** *J Immunol* 2003, **170**:2993–3001.
35. Pabón A, Carmona J, Burgos LC, Blair S: **Oxidative stress in patients with non-complicated malaria.** *Clin Biochem* 2003, **36**:71–78.
36. Luty AJ, Perkins DJ, Lell B, Schmidt-Ott R, Lehman LG, Luckner D, Greve B, Matousek P, Herbich K, Schmid D, Weinberg JB, Kremsner PG: **Low interleukin-12 activity in severe *Plasmodium falciparum* malaria.** *Infect Immun* 2000, **68**:3909–15.
37. Sinha S, Qidwai T, Kanchan K, Jha GN, Anand P, Pati SS, Mohanty S, Mishra SK, Tyagi PK, Sharma SK, Awasthi S, Venkatesh V, Habib S: **Distinct cytokine profiles define clinical immune response to falciparum malaria in regions of high or low disease transmission.** *Eur Cytokine Netw* 2010, **21**:232–240.
38. Cabantous S, Poudiougou B, Oumar AA, Traore A, Barry A, Vitte J, Bongrand P, Marquet S, Doumbo O, Dessein AJ: **Genetic evidence for the aggravation of *Plasmodium falciparum* malaria by interleukin 4.** *J Infect Dis* 2009, **200**:1530–1539.
39. Carvalho LH, Sano G-I, Hafalla JCR, Morrot A, de Lafaille MA C, Zavala F: **IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages.** *Nat Med* 2002, **8**:166–170.
40. Morrot A, Hafalla JCR, Cockburn IA, Carvalho LH, Zavala F: **IL-4 receptor expression on CD8+ T cells is required for the development of protective memory responses against liver stages of malaria parasites.** *J Exp Med* 2005, **202**:551–560.
41. Zhu J, Yamane H, Cote-Sierra J, Guo L, Paul WE: **GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors.** *Cell Res* 2006, **16**:3–10.

doi:10.1186/1475-2875-12-69
Cite this article as: Mendonça *et al.*: Networking the host immune response in *Plasmodium vivax* malaria. *Malaria Journal* 2013 **12**:69.

5.4 MANUSCRITO IV

***DDX39B (BAT1)*, *TNF* and *IL6* gene polymorphisms and association with clinical outcomes of patients with *Plasmodium vivax* malaria.**

Este estudo analisa a associação entre SNPs em genes relacionados a resposta imune (*DDX39B*, *TNF* e *IL6*), mediadores inflamatórios plasmáticos (IL-6, TNF, CXCL10 e proteína C reativa) e os desfechos clínicos da malária vivax.

Resumo dos resultados: O alelo G do polimorfismo *DDX39B*-22C>G foi associado com a manifestação clínica amena da malária, enquanto que o alelo C foi um fator de risco para complicações da doença. Participantes heterozigotos para *TNF*-308 (GA) e *DDX39B*-348 (CT) tiveram níveis plasmáticos maiores de TNF do que os homozigotos selvagens. Indivíduos com a combinação de genótipos GC/CC/GG/GG e GG/CT/GG/GG (*DDX39B*-22/*DDX39B*-348/*TNF*-308/*IL6*-176) tiveram um risco diminuído e aumentado para malária sintomática, respectivamente, quando comparados com o grupo assintomático e não-infectado. A combinação de genótipos (GC/CC/GG/GG) associada com risco maior para malária foi associada com níveis séricos diminuídos de TNF e IL-6.

Este trabalho foi publicado no periódico internacional *Malaria Journal.*

MALARIA JOURNAL

RESEARCH Open Access

DDX39B (*BAT1*), *TNF* and *IL6* gene polymorphisms and association with clinical outcomes of patients with *Plasmodium vivax* malaria

Vitor RR Mendonça[1,2], Ligia CL Souza[1,2], Gabriela C Garcia[1], Belisa ML Magalhães[3], Marcus VG Lacerda[3], Bruno B Andrade[4], Marilda S Gonçalves[1,2] and Manoel Barral-Netto[1,2,5*]

Abstract

Background: *DDX39B (BAT1)* encodes an RNA helicase known to regulate expression of TNF and IL-6. Elevated levels of these two cytokines are associated with increased severity of clinical malaria. The aim of this study was to investigate the relationship between single nucleotide polymorphisms (SNPs) in the *DDX39B*, *TNF* and *IL6* genes and the clinical outcomes of patients with *Plasmodium vivax* malaria.

Methods: Cross-sectional investigations were carried out in two regions of the Brazilian Amazon where several studies on the pathogenesis of vivax malaria had been performed. Individuals were categorized according to infection status as well as clinical presentation into the following groups: uninfected, asymptomatic infection, mild infection, or complicated infection. Polymorphisms were identified using PCR restriction fragment-length polymorphism analysis and the restriction enzymes *Nla*III or *Nco*I. The plasma levels of cytokines were determined using ELISA.

Results: The G allele of *DDX39B*-22C > G was associated with absent or decreased manifestations of malaria and the C allele was a risk factor for disease complications. Study participants heterozygous for *TNF-308* (GA) and *DDX39B-348* (CT) had higher TNF levels than wild-type participants. Haplotypes that included *DDX39B* (−22C > G and -348C > T) and *TNF* polymorphisms were not directly associated with mild or complicated malaria infections; however, haplotypes AGC, ACC, GGT, AGT and ACT were associated with increased TNF levels. Participants with genotype combinations GC/CC/GG/GG and GG/CT/GG/GG (*DDX39B-22/DDX39B-348/TNF-308/IL6-176*) had decreased and increased risk of mild malaria, respectively, compared with asymptomatic and uninfected participants. GC/CC/GG/GG was linked to decreased TNF and IL-6 levels.

Conclusions: This is the first study to describe patients with *DDX39B* and *IL6* SNPs who had vivax malaria. These findings support the postulation that a set of mutations in immune-related genes is associated with inflammatory mediators and the clinical outcomes of patients with malaria.

Keywords: *DDX39B* (*BAT1*), Single nucleotide polymorphisms, Immune response, *Plasmodium vivax*, Malaria

Background

Plasmodium vivax malaria is a major worldwide threat, with two to three billion people remaining at risk of infection [1]. The clinical outcomes of patients with vivax malaria range from asymptomatic infection to complicated, and potentially lethal disease [2]. The risk for progression of severe *Plasmodium falciparum* malaria has been thought to be partly accounted for by host genetic factors [3]. These genetic factors are likely to involve several genetic alterations in immune mediators as well as molecules involved in mechanisms of cytoadherence and haemoglobinopathies [4]. Understanding the key host genetic determinants of susceptibility to malaria is critical for developing better therapies and vaccine strategies.

The nuclear protein HLA-B-associated transcript 1 (BAT1) is an RNA helicase encoded by the *DDX39B* gene (DEAD [Asp-Glu-Ala-Asp] box polypeptide 39B, also

* Correspondence: mbarral@bahia.fiocruz.br
[1]Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Brazil
[2]Faculdade de Medicina, Universidade Federal da Bahia, Salvador, Brazil
Full list of author information is available at the end of the article

BioMed Central

© 2014 Mendonça et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

known as *BAT1*). BAT1 has been described as a negative regulator of inflammation by modulating expression of proinflammatory cytokines, such as TNF and IL-6, which suggests that it plays a protective role in several immune-mediated disorders [5]. The -22C > G and -348C > T polymorphisms in the promoter region of *DDX39B* have been shown to affect its transcriptional activity and the binding of nuclear transcription factors such as YY1 and Oct1 oligonucleotides at the respective positions −348 and −22 relative to the transcription start site of *DDX39B* [6]. TNF (=TNFalpha or TNFA) and IL-6 are key mediators associated with malaria symptoms, and their levels are increased in proportion to the severity of disease [7-12]. The hypothesis that *DDX39B* may have an effect on the clinical presentation of malaria by its modulation of the expression of proinflammatory cytokines involved in the pathogenesis of the disease is appealing, but has not yet been tested.

Single nucleotide polymorphisms (SNPs) in the *TNF* (−308G > A) and *IL6* (−176G > C) genes may regulate the plasma levels of these cytokines; however, the mechanism of regulation is not yet fully understood [13-15]. Given the major role of the host immune system in *P. vivax* infection, the aim of this study was to determine whether mutations in the *DDX39B*, *TNF* and *IL6* genes were associated with the clinical outcomes of patients with vivax malaria. The frequency of SNPs in *DDX39B* (−22C > G and -348C > T), *TNF* (−308G > A) and *IL6* (−176G > C) were compared between *P. vivax*-infected study participants who exhibited different clinical outcomes, including asymptomatic infection, mild malaria, complicated malaria, and no infection. Associations between these immune-related mutations and plasma levels of TNF, IL-6, C-X-C motif chemokine 10 (CXCL10), and C-reactive protein (CRP) were also tested. The results reported here revealed that a combination of *DDX39B, TNF* and *IL6* host genotypes were associated with manifestations of malaria, mainly by altering plasma levels of TNF and IL-6.

Methods

Study participants

This report describes series of patients from two distinct studies. The first study performed retrospective analyses of cryopreserved heparinized blood samples from participants living in riverine communities of the state of Rondônia, in the Brazilian Western Amazon, who were recruited between 2006 and 2007, as previously described [7,16-23]. Malaria was diagnosed using two methods: 1) microscopic examination of a thick blood smear performed by professionals at the Brazilian National Foundation of Health (FUNASA); and 2) polymerase chain reaction (PCR) performed at the Oswaldo Cruz Foundation (FIOCRUZ), Salvador, Brazil, as previously described [16-18]. The study included individuals who had been living in the endemic area for more than six months. Exclusion criteria included conditions known to interfere with the parameters evaluated in this report, such as coinfections and chronic diseases: *P. falciparum* infection confirmed by nested PCR; documented or referred viral hepatitis (hepatitis A, B, C, D virus [HAV, HBV, HCV, HDV]); chronic alcoholism; human immunodeficiency virus (HIV) infection; yellow fever; dengue; leptospirosis; tuberculosis; Hansen disease; visceral leishmaniasis; cancer and/or other chronic degenerative disease; sickle cell trait; and the use of hepatotoxic and immunosuppressant drugs. A total of 257 participants were enrolled in this first part of the study. As reported previously, all asymptomatic participants infected with *P. vivax* who were identified by active case detection were monitored for 30 days for the evaluation of malaria manifestations [16-18]. Participants who were positive for *P. vivax* infection but remained without acute febrile signs for 30 days were considered to be asymptomatic cases. Those individuals with positive parasitaemia and with mild symptoms were considered to have mild vivax malaria. Thus, the individuals from this region were divided into three groups as follows: mild malaria (n = 76), asymptomatic malaria (n = 104) and uninfected controls (n = 77). All symptomatic cases were treated following the guidelines of the National Foundation of Health, Brazil, and received chloroquine for three days and primaquine (0.5 mg/kg/day) for seven days. The asymptomatic carriers were treated after the monitoring period, as reported previously [17]. This first part of the study was approved by the Ethics Committee of the São Lucas University, Rondônia, Brazil; and all participants provided written informed consent.

Participants with complicated *Plasmodium vivax* malaria

Because there was a small number and unavailable blood samples of malaria cases (n = 9) with signs/symptoms of complicated disease among the first series of patients recruited in Rondonia, in order to evaluate the role of *DDX39B (BAT1)* polymorphism in complicated cases of vivax malaria, a second series of patients (second part of this report) was recruited from the state of Amazonas in the Western Brazilian Amazon between 2009 and 2013. Individuals of all ages who were hospitalized with an unidentified acute febrile syndrome at the reference hospital from the Fundação de Medicina Tropical Dr Heitor Vieira Dourado (FMT-HVD), Amazonas, Brazil, were tested for malaria using microscopic examination of a thick blood thick smear, and those with PCR-confirmed *P. vivax* were recruited. Patients were excluded for the following conditions: microscopic or molecular diagnosis of *P. falciparum* or *P. vivax* and *P. falciparum* malaria (mixed infection), serologic diagnosis of viral hepatitis (HAV, HBV, HCV, and HDV), HIV, or leptospirosis. Patients with vivax malaria with primaquine-induced haemolysis (patients taking primaquine with decreasing haemoglobin levels

to <10 g/dL and reticulocyte counts >1.5%, or increased indirect bilirubin levels after primaquine treatment) were also excluded. The study participants from this second series had either mild signs/symptoms of acute malaria (mild malaria, n = 69) or clinical complications, as listed in Table 1 (complicated vivax malaria, n = 31). Complicated vivax malaria was defined according to the criteria for severe malaria from the World Health Organization (WHO) or based on the presence of hyperbilirubinaemia (serum total bilirubin > 51.3 μmol/L) [24]. Although all the participants in this second study were PCR positive, some of these study patients with complicated malaria (just for this particular group) had already started therapy for malaria before blood samples were taken, which might have affected the results of the laboratory and parasitaemia evaluations shown in Table 1. This second part of the study was approved by the Ethics Committee of the FMT-HVD, and all participants provided written informed consent.

Table 1 Characteristics of the second series of participants with complicated vivax malaria: Amazonas, Brazil

			Clinical presentation at admission						
Patient no.	Gender	Age (years)	Major manifestation	Secondary manifestation	Previous treatment	Parasitaemia	Disease duration (days)	Haemoblogin (g/dL)	Total bilirubin (μmol/L)
1	M	59	hyperbilirubinaemia		No	6,222	7	16.9	403.56
2	F	38	hyperlactemia		Yes	0	10	10.9	30.78
3	M	31	hyperbilirubinaemia		No	3,298	10	11.0	54.72
4	F	37	hyperbilirubinaemia		No	67,467	7	8.9	51.30
5	M	18	hyperbilirubinaemia		No	1,848	4	13.5	165.87
6	M	45	severe anaemia	hyperbilirubinaemia	No	243	10	6.5	138.51
7	F	40	severe anaemia		No	33,553	10	7.0	27.36
8	M	66	convulsion		Yes	185	14	13.0	*
9	M	17	hyperbilirubinaemia		No	226	6	11.9	53.01
10	M	31	hyperbilirubinaemia		No	5,937	15	13.5	165.87
11	M	1	severe anaemia		No	38,712	9	5.0	8.55
12	M	26	hyperbilirubinaemia		No	128	2	10.6	61.56
13	F	4 months	convulsion	prostration	No	108,033	7	11.7	*
14	M	19	severe anaemia		No	0	17	6.5	15.39
15	M	50	respiratory failure		No	310	5	10.1	8.55
16	F	35	severe anaemia	hyperbilirubinaemia	No	34,673	1	4.8	138.51
17	F	41	respiratory failure		No	24,815	8	9.9	39.33
18	F	18	hyperbilirubinaemia		No	670	5	11.7	61.56
19	M	44	severe anaemia		Yes	0	7	6.9	13.68
20	F	23	severe anaemia		Yes	264	8	6.4	6.84
21	M	5	prostration		No	291	15	8.2	6.84
22	M	21	hyperbilirubinaemia		Yes	832	7	9.7	102.6
23	M	12	respiratory failure		Yes	0	6	7.8	15.39
24	F	74	respiratory failure		No	325	5	11.7	29.07
25	M	37	respiratory failure		Yes	0	10	7.1	17.10
26	F	26	hyperbilirubinaemia		No	254	9	11.3	140.22
27	M	53	hyperbilirubinaemia		No	45,833	6	11.8	59.85
28	F	36	hyperbilirubinaemia		No	0	3	7.9	85.50
29	F	15	severe anaemia		Yes	8,894	8	6.9	11.97
30	M	22	hyperbilirubinaemia		No	3,054	7	15.1	73.53
31	M	25	hyperbilirubinaemia		Yes	27,954	3	15.0	138.51

Severe anaemia was defined as haemoglobin levels below 7 g/dL for adults and below 5 g/dL for children and hyperbilirubinaemia by serum total bilirubins >51.3 μmol/L. Respiratory failure was defined as tachypnea, shortness of breath, mental confusion clinical signs of hypoxaemia (central and/or peripheral cyanosis). Previous treatment indicates participants who already started malaria therapy before the blood sample collection. *Total bilirubin was not measured in these individuals.

Genotyping

DNA was extracted from 200 μL of peripheral blood using a standard Qiagen DNA blood mini kit (Valencia, CA, USA) according to the manufacturer's protocol. The SNPs at positions −22 (C > G) (rs2239527; G ancestral allele) and −348 (C > T) (rs2239528 ;C ancestral allele) in the promoter region of *DDX39B* and at position −176 of *IL6* (G > C) (rs2234683; G ancestral allele) were typed using PCR restriction fragment-length polymorphism analysis with the restriction enzyme *Nla*III (New England Biolabs), according to protocols previously published by Ramasawmy *et al.* [25] and Yalcin *et al.* [26], respectively. *TNF-308* (G > A) (rs1800629; G ancestral allele) polymorphism was also evaluated using PCR restriction fragment-length polymorphism analysis with the restriction enzyme *Nco*I (New England Biolabs), as published previously [27]. *DDX39B* and *TNF* PCR products were electrophoresed on 10% polyacrylamide gels and *IL6* products were separated by electrophoresis on 1% agarose gels under nondenaturing conditions. The PCR products were then detected by staining with ethidium bromide and visualized under ultraviolet illumination.

Plasma measurements

The plasma levels of IL-6, CXCL10, and TNF were measured using a cytometric bead array system (BD Biosciences Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. CRP levels in plasma were measured using the turbidimetric immunoassay method, performed at the Federal University of Bahia and Faculdade São Lucas, Brazil.

Statistical analysis

Categorized variables (genotypes, alleles, haplotypes, and genotype combinations) were compared using the Chi-square test or Fisher exact test in 2x2 contingency tables along with the relevant odds ratio (OR) and 95% confidence interval (CI). Univariate linear regression analysis was performed to assess the associations between combinations of genotypes and malaria symptomatology. Ordinal variables were evaluated using the Mann–Whitney (between two groups) or Kruskal-Wallis test followed by the Dunn multiple comparison test or trend analysis (when more than two groups were compared). Hardy-Weinberg equilibrium (HWE) was assessed for the different groups by comparing the observed number of different genotypes with those expected under HWE for the estimated allele frequency. The power of this study was calculated based on a medium effect size, a significance level of 0.05, and four degrees of freedom (Chi-square test). A power of 98.3% was found for the first series of participants (n = 257) and a power of 71.10% for the second series of participants (n = 110). Statistical analyses were performed using GraphPad Prism (version 5.0b) software (GraphPad Software, San Diego, CA, USA) or R version 2.15.1 (The R Foundation for Statistical Computing, Vienna, Austria).

Results

Baseline characteristics of first series of participants

All different groups of vivax malaria infection had a slightly majority of women. Participants with asymptomatic malaria were older than the uninfected participants and those with mild malaria ($P = 0.0264$; Table 2). Participants with asymptomatic malaria reported a higher number of previous infections (median 15.0, interquartile range (IQR) 12.00-18.75) than the uninfected participants or those with mild infection ($P < 0.0001$; Table 2). In addition, participants with asymptomatic *P. vivax* infection reported living for a longer time in the endemic area (71.15% more than ten years) than those in the other groups ($P = 0.0030$; Table 2). These results were expected, since this set of patients was a subsample of a larger cohort of individuals where similar results were found [7,16-23].

Single nucleotide polymorphisms

Genotype and allele distributions of *IL6*-176G > C and *TNF*-308G > A polymorphisms were compared in the main clinical groups and in participants stratified according to mild *vs* asymptomatic vivax malaria infection, infected *vs* uninfected and asymptomatic *vs* uninfected (Table 3). No association was found between alleles or genotypes of *IL6* and *TNF* SNPs and the different clinical outcomes of vivax malaria (Table 3). The distributions of *IL6*-176G > C and *TNF*-308G > A genotypes in all categories were under HWE. Table 4 shows the results for *DDX39B* polymorphisms (−22C > G and -348C > T) according to the same categories of participants (Table 4). No differences were observed for the SNPs *DDX39B-22C > G* and *DDX39B-348C > T* with regard to mild malaria, asymptomatic malaria, or infection status. The distribution of *DDX39B*-22C > G in the asymptomatic participants was not under HWE ($x2 = 6.10$, $P = 0.0134$), suggesting those participants reflect evolutionary selective pressure. The frequencies of *DDX39B* genotypes were under HWE in all the other categories of vivax malaria infection.

Association of single nucleotide polymorphisms with inflammatory mediators levels

The SNPs were assessed regarding association with systemic levels of TNF, IL-6, CXCL10, and CRP, which have been associated with vivax malaria manifestations [23]. Participants with the *TNF*-308 GA genotype or A allele had higher levels of TNF than those with the GG genotype or G allele ($P = 0.0347$ and $P = 0.0296$, respectively, Figure 1A,B). Participants with the *DDX39B*-348 CT genotype or T allele had higher concentrations of TNF than those with the CC genotype or C allele

Table 2 Baseline characteristics of the first series of participants enrolled in the first part of the study: Rondonia, Brazil

	Uninfected (n = 77)	Asymptomatic vivax malaria (n = 104)	Mild vivax malaria (n = 76)	*P* value
Male - no. (%)	36 (46.75)	47 (45.19)	36 (47.37)	0.9548**
Median (IQR*) age (yr)	35.00 (25.50-45.00)	42.00 (32.00-49.00)	36.00 (27.25-50.00)	0.0264***
Median (IQR*) of previous malaria episodes	12.00 (6.00-17.00)	15.00 (12.00-18.75)	6.00 (1.00-12.75)	<0.0001***
Time residing in the area (yr)				
≤2	17 (22.08)	24 (23.08)	24 (31.58)	0.0011**
3 to 10	19 (24.67)	6 (5.77)	16 (21.05)	
>10	41 (53.25)	74 (71.15)	36 (47.37)	

* IQR, interquartile range; **Categorized variables were compared using Chi-square test; ***Ordinal variables were compared using the Kruskal-Wallis test with the Dunn multiple comparison test

($P = 0.0215$ and $P = 0.0299$, respectively, Figure 1A,B). Polymorphism at *DDX39B*-22 appeared to correlate with serum concentrations of CRP and CXCL10, but not IL-6. Participants with the *DDX39B*-22 CC genotype exhibited higher levels of CRP than those with the GC genotype ($P = 0.0395$, Figure 1C) but this difference was not observed in the allele analysis ($P = 0.0622$, Figure 1D). No relevant associations were found between genotypes or alleles of *DDX39B*-22 C > G and plasma IL-6 levels ($P = 0.0496$ and $P = 0.7475$, respectively, Figure 1E,F). Furthermore, participants carrying the *DDX39B*-22 CG genotype had lower CXCL10 levels than those with the GG genotype ($P = 0.0294$, Figure 1E), although no difference was seen in the allele analysis ($P = 0.0898$, Figure 1F).

Haplotypes and combinations of genotypes

DDX39B and *TNF* are located in the same major histocompatibility complex (MHC) region on chromosome 6, and therefore assessment of the association of these polymorphisms with the outcomes of malaria can be performed using haplotype analysis. The assessment of haplotypes representing all possible combinations of *TNF*-308, *DDX39B*-22 and *DDX39B*-348 SNPs was unable to identify significant association with any of the clinical categories of vivax malaria infection (Figure 2A). Nevertheless, haplotypes were associated with differential expression of inflammatory mediators in plasma, which could potentially influence the degree of immunopathology and malaria manifestations. *TNF*-308, *DDX39B*-22, and *DDX39B*-348

Table 3 *TNF* (−308G > A) and *IL-6* (−176G > C) polymorphisms and outcome of vivax malaria infection: first series of participants from Rondonia, Brazil

	Genotype			χ2	Allele frequency		χ2	GG *vs* GA + AA χ2
	GG n (%)	GA n (%)	AA n (%)		G (%)	A (%)		
***TNF-308* polymorphism**								
Uninfected (n = 77)	60 (77.92)	15 (19.48)	2 (2.60)	2.12 p = 0.7134#	135 (87.66)	19 (12.34)	0.57 p = 0.7509	0.96 p = 0.6178
Asymptomatic malaria (n = 104)	78 (75.00)	25 (24.04)	1 (0.96)		181 (87.02)	27 (12.98)		
Mild malaria (n = 76)	54 (71.05)	21 (27.63)	1 (1.32)		129 (84.87)	23 (15.13)		
Infected *vs* uninfected				p = 0.3401*#			p = 0.6745*	P = 0.5316*
Mild *vs* asymptomatic				p = 0.6059*#			p = 4798*	P = 0.4323*
Asymptomatic *vs* uninfected				p = 0.5866*#			p = 8749*	P = 0.7252*
	Genotype			**χ2**	**Allele frequency**		**χ2**	**GG *vs* GC + CC χ2**
	GG n (%)	**GC n (%)**	**CC n (%)**		**G (%)**	**C (%)**		
***IL6-176* polymorphism**								
Uninfected (n = 77)	49 (63.64)	23 (29.87)	5 (6.49)	2.16 p = 0.7058	121 (78.57)	33 (21.43)	1.61 p = 0.4462	1.91 p = 0.3854
Asymptomatic malaria (n = 104)	60 (57.69)	38 (36.54)	6 (5.77)		158 (75.96)	50 (24.04)		
Mild malaria (n = 76)	40 (52.63)	30 (39.47)	6 (7.90)		110 (72.37)	42 (27.63)		
Infected *vs* uninfected				1.56 p = 0.4579			p = 0.3695*	p = 0.2702*
Mild *vs* asymptomatic				1.30 p = 0.5214			p = 0.2618*	p = 0.2711*
Asymptomatic *vs* uninfected				0.88 p = 0.6435			p = 0.0819*	p = 0.4458*

*In these cases, Fisher exact test was used. #Analysis excluded genotype AA. χ2: coefficient and *P* value measured using Chi-square test. Infected individuals represent symptomatic plus asymptomatic cases.

Table 4 *DDX39B* polymorphisms (−22C > G and -348C > T) and outcome of vivax malaria infections: first series of participants from Rondonia, Brazil

	Genotype			χ2	Allele frequency		χ2	CC *vs* CG + GG χ2
	CC n (%)	CG n (%)	GG n (%)		C (%)	G (%)		
***DDX39B-22* polymorphism**								
Uninfected (n = 77)	10 (12.99)	38 (49.35)	29 (37.66)	6.75 p = 0.1496	58 (37.66)	96 (62.34)	2.35 p = 0.3088	0.53 p = 0.7660
Asymptomatic malaria (n = 104)	15 (14.42)	64 (61.54)	25 (24.04)		94 (45.19)	114 (54.81)		
Mild malaria (n =7 6)	13 (17.10)	34 (44.74)	29 (38.16)		60 (39.47)	92 (60.53)		
Infected *vs* uninfected				1.48 p = 0.4758			p = 0.3281*	p = 0.2460*
Mild *vs* asymptomatic				2.90 p = 0.2339			p = 0.6244*	p = 0.7028*
Asymptomatic *vs* uninfected				3.98 p = 0.1364			p = 0.1625*	p = 0.8307*
	Genotype				**Allele frequency**			**CC *vs* TC + TT χ2**
	CC n (%)	**TC n (%)**	**TT n (%)**		**C (%)**	**T (%)**		
***DDX39B-348* polymorphism**								
Uninfected (n = 77)	64 (83.12)	12 (15.58)	1 (1.30)	1.99 p = 0.3691[#]	140 (90.91)	14 (9.09)	1.00 p = 0.6041	1.56 p = 0.4586
Asymptomatic malaria (n = 104)	83 (79.81)	21 (20.19)	0 (0.00)		187 (89.90)	21 (10.10)		
Mild malaria (n = 76)	57 (75.00)	19 (25.00)	0 (0.00)		133 (87.50)	19 (12.50)		
Infected *vs* uninfected				p = 0.2397*[#]			p = 0.5342*	p = 0.4012*
Symptomatic *vs* asymptomatic				p = 0.2372*[#]			p = 0.3469*	p = 0.3108*
Asymptomatic *vs* uninfected				p = 0.5594*[#]			p = 0.8578*	p = 0.7008*

*In these cases, Fisher exact test was used. [#]Analysis excluded genotype TT. χ2: coefficient and *P* value measured using Chi-square test. Infected individuals represent symptomatic plus asymptomatic cases.

haplotypes GGC and GCC were linked with lower concentrations of CRP and CXCL10, respectively, than other haplotypes ($P = 0.0246$, Figure 2B; $P = 0.0071$, Figure 3C; respectively). Intriguingly, several haplotypes were associated with increased plasma TNF levels (Figure 2D). Thus, because they were found to be associated with elevated or decreased levels of CRP, CXCL10, and TNF, haplotypes may be associated with the outcomes of vivax malaria infection.

Analysis of combinations of genotypes was performed to determine association with manifestations of vivax malaria. Participants with *DDX39B*-22/*DDX39B*-348/*TNF*-308/*IL6*-176 genotype combinations GC/CC/GG/GG and GG/CT/GG/GG had decreased and increased risk, respectively, of developing manifestations of malaria relative to asymptomatic participants (OR 0.41, 95% CI 0.17-0.98, $P = 0.0428$; OR 4.77, 95% CI 1.10-20.59, p = 0.0361; respectively, Figure 3A). Moreover, the genotype combination GC/CC/GG/GG was associated with lower TNF and IL-6 levels than other genotypes ($P = 0.0070$ and $P = 0.0057$, respectively; Figure 3B,C), suggesting that this set of genotypes may protect against more severe malaria because of association with reduced levels of inflammatory cytokines.

Second series of study participants: *DDX39B* polymorphisms and complicated vivax malaria

An additional aim of this combined study was to specifically evaluate the association between *DDX39B* polymorphisms and complicated *P. vivax* malaria. Patients screened in a reference hospital from the Brazilian Amazon who manifested complicated malaria were compared with those presenting with mild disease. Participants with mild malaria had a median age of 36 years (IQR 27–46) and 81.16% were male (n = 56). Participants with complicated disease had a similar age (median 31 years, IQR 18–41; $P = 0.0563$) and there was a slightly lower proportion of males (19 of 31 [61.29%], $P = 0.0460$). Characteristics of the patients with complicated malaria are shown in Table 1. Interestingly, the patients in this study who were categorized with complicated vivax malaria exhibited more often hyperbilirubinaemia (serum total bilirubin >51.3 μmol/L), which has been reported to be a common complication of patients with vivax malaria [28,29].

There was significant difference in the distribution frequency of the polymorphism *DDX39B*-22C > G in the participants with mild versus complicated malaria (x2 = 6.72, $P = 0.0347$). The proportion of -22C homozygosity among patients with complicated and mild malaria was 25.80% (n = 8) and 7.25% (n = 5), respectively (Table 5). Participants were categorized based on the presence or absence of the G allele (genotypes GG and GC *vs* genotype CC) and the G allele was significantly more frequent in patients with mild vivax malaria than in patients with complicated infection ($P = 0.0207$, Table 5). No differences were seen for the polymorphism *DDX39B* (−348C > T) regarding the distribution of genotypes and alleles in patients with mild

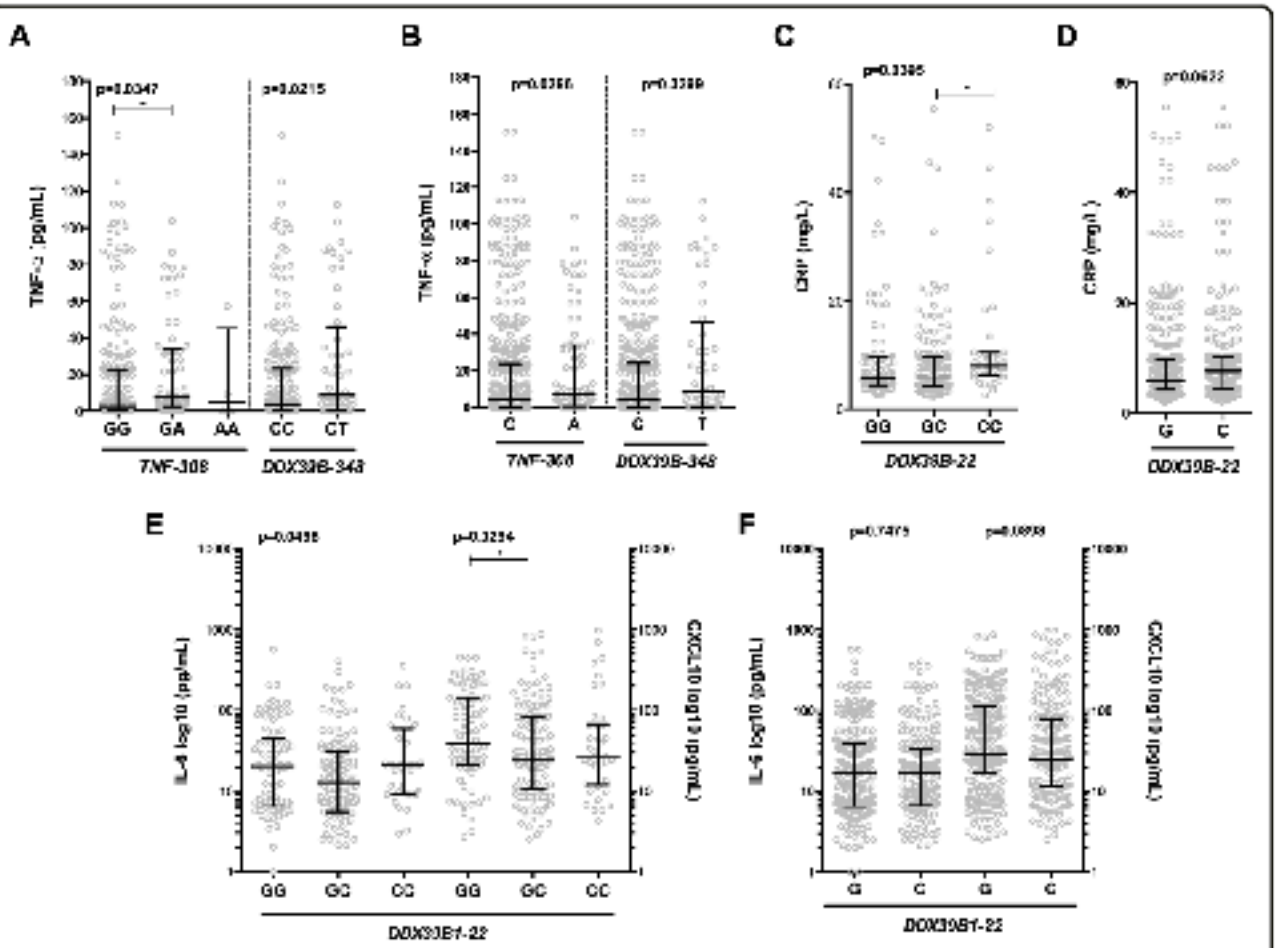


Figure 1 Influence of genetic alterations on levels of inflammatory mediators. Each symbol represents a single patient, and the lines represent medians and interquartile range. **(A)** Levels of TNF according to genotypes of *TNF*-308G > A and *DDX39B*-348C > T polymorphisms. Analysis excluded TT (*DDX39B*-348C > T), because only 1 uninfected participant had this genotype. **(B)** Levels of TNF according to alleles of *TNF*-308G > A and *DDX39B*-348C > T polymorphisms. **(C)** CRP plasma levels according to different *DDX39B*-22C > G genotypes. **(D)** CRP plasma levels according to different *DDX39B*-22C > G alleles. **(E)** IL-6 and CXCL10 levels according to *DDX39B*-22C > G genotypes. **(F)** IL-6 and CXCL10 levels according to *DDX39B*-22C > G alleles. The data were compared using the Mann–Whitney test (comparisons between two groups), the Kruskal-Wallis test with Dunn multiple comparisons or linear trend analysis (comparisons between more than 2 groups). *P* values are shown in each panel.

and complicated malaria (Table 5). The distribution of *DDX39B*-22C > G genotypes in the patients with mild malaria was not under HWE (x2 = 5.22, *P* = 0.0222). The *DDX39B* genotypes were under HWE in the patients with complicated malaria.

Discussion

Immunity of the human host to malaria is likely to be mediated by T-cell recognition of *Plasmodium sp.* epitopes on infected host cells via class I and II MHC antigens [30]. Given the importance of the MHC to the immune response, genetic studies of the human MHC I have correlated polymorphisms in this region with susceptibility to malaria. The alleles A*30:01 and A*33:01 of MHC I were found to be associated with malaria severity in Mali [30-32]. The *DDX39B (BAT1)* gene is located on chromosome 6 near the *TNF* gene in the same MHC region and seems to influence expression of several immune-related genes [6]. This study found that the C allele of *DDX39B*-22C > G is a potential risk factor of complicated vivax malaria in the Brazilian Amazon. This finding may be expected, as this allele has been associated with reduced binding to transcription factors and expression of inflammatory cytokines [5]. Haplotype analysis (including *DDX39B* and *TNF* polymorphisms) found that genetic alterations in these immune-mediated genes may influence disease outcome by altering TNF plasma levels. In addition, the genotype combinations GC/CC/GG/GG and GG/CT/GG/GG, corresponding to the respective polymorphisms *DDX39B*-22/*DDX39B*-348/*TNF*-308/*IL6*-176, were associated with a decreased or increased risk, respectively, of developing mild vivax malaria, probably by altering TNF and IL-6 levels. To the best of our knowledge, this is the first report assessing the relationship between *DDX39B* polymorphisms and

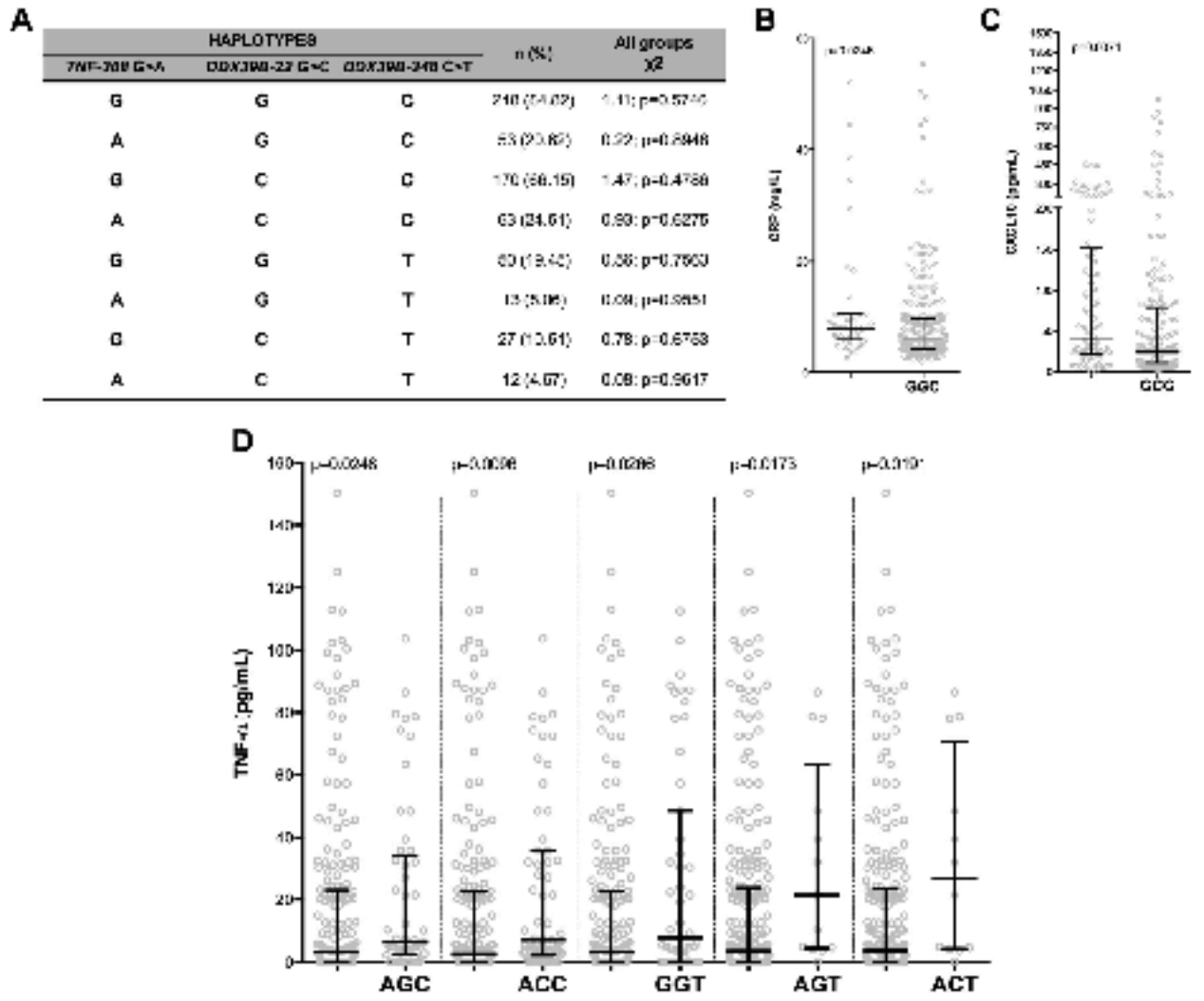


Figure 2 Haplotype influence on inflammatory mediators and outcomes of malaria infection. Each symbol represents a single patient, and the lines represent medians and interquartile range. **(A)** The figure represents all the possible haplotypes for *TNF*-308G > A, *DDX398*-348C > T, and *DDX398*-22C > G polymorphisms. Haplotypes were compared between the 3 categories of vivax malaria infections (uninfected, asymptomatic, mild malaria). **(B)** CRP levels in participants with GGC haplotype compared to individuals with other haplotypes. **(C)** CXCL10 plasma concentration in participants with GCC haplotype compared to individuals with other haplotypes. **(D)** TNF levels in participants with different haplotypes. Each haplotype was compared with participants with other haplotypes. The differences between groups illustrated in **(A)** were compared using the chi-square test. The data in **(B)**, **(C)**, and **(D)** were compared using the Mann–Whitney test. *P* values are shown in each panel.

malaria outcomes, and also one of the few studies to analyze the manifestations of *P. vivax* infections in relation to combinations of immune-related genotypes.

Plasma concentrations of IL-6 have been found to be associated with severe disease and death from malaria, and the *IL6*-176C allele was associated with increased expression of IL-6 in neonates and adults developing acute phase reactions [14,15,33,34]. A study of sympatric ethnic groups in Mali found that the frequency of *IL6* CG/GG genotypes was higher in non-Fulani ethnic groups, who have increased susceptibility to malaria, in both symptomatic and asymptomatic falciparum malaria cases [35]. However, in our study, there were no differences in the distribution of *IL6*-176G > C in the participants making up the different clinical groups of vivax malaria, which may indicate that this polymorphism plays different roles in *P. vivax* infection and/or in a Brazilian population. Genetic changes in *TNF* have been described in several studies of different populations throughout the world, and there have sometimes been contradictory results [23]. The SNP *TNF*-308G > A has been associated with increased susceptibility, resistance or there has been no association with severity to malaria caused by *P. falciparum* [36-39]. A study of a population in the Brazilian Amazon that was similar to our population found that one *TNF* haplotype (*TNF*-1031T/-863A/-857T/-308G/-238G) including the *TNF*-308G allele was associated

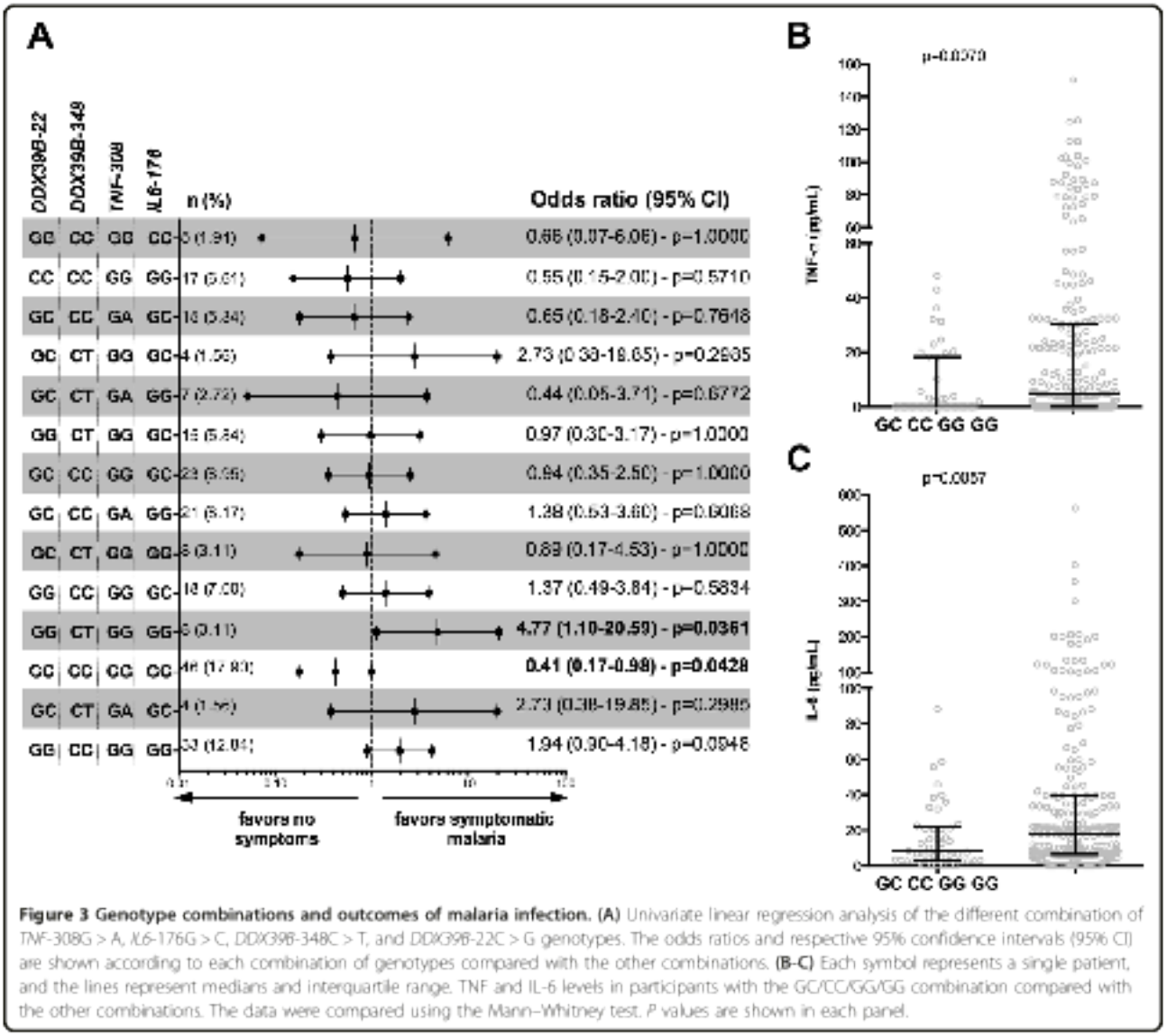


Figure 3 Genotype combinations and outcomes of malaria infection. (A) Univariate linear regression analysis of the different combination of *TNF*-308G > A, *IL6*-176G > C, *DDX39B*-348C > T, and *DDX39B*-22C > G genotypes. The odds ratios and respective 95% confidence intervals (95% CI) are shown according to each combination of genotypes compared with the other combinations. **(B-C)** Each symbol represents a single patient, and the lines represent medians and interquartile range. TNF and IL-6 levels in participants with the GC/CC/GG/GG combination compared with the other combinations. The data were compared using the Mann–Whitney test. *P* values are shown in each panel.

Table 5 *DDX39B* polymorphisms (−22C > G and -348C > T) and complicated vivax malaria infections: second series of participants from Amazonas, Brazil

	Genotype			χ2	Allele frequency		χ2	CC *vs* CG + GG χ2
	CC n (%)	CG n (%)	GG n (%)		C (%)	G (%)		
***DDX39B-22* polymorphism**								
Mild malaria (n = 69)	5 (7.25)	41 (59.42)	23 (33.33)	**6.72 p = 0.0347**	51 (36.96)	87 (63.04)	p = 0.0628*	**p = 0.0207***
Complicated malaria (n = 31)	8 (25.80)	16 (51.61)	7 (22.58)		32 (51.61)	30 (48.39)		
	Genotype				Allele frequency			CC *vs* TC + TT χ2
	CC n (%)	TC n (%)	TT n (%)		C (%)	T (%)		
***DDX39B-348* polymorphism**								
Mild malaria (n = 69)	55 (79.71)	14 (20.29)	0 (0.00)	p = 7,850*#	124 (89.86)	14 (10.14)	p = 0.7965*	p = 7,850*
Complicated malaria (n = 31)	26 (83.87)	5 (16.13)	0 (0.00)		57 (91.93)	5 (8.07)		

*In these cases, Fisher exact test was used. #Analysis excluded genotype TT. χ2: coefficient and *P* value measured using Chi-square test.

with increased susceptibility to mild vivax malaria [40]. Our study did not find an association between *TNF*-308G and clinical manifestations of malaria. It is noteworthy that *TNF*-308G > A may not be a causal mutation, and that this polymorphism could be in linkage disequilibrium with other causal mutations located close to the *TNF* gene [41]. Thus, this polymorphism may have a moderate effect or may have an epistatic effect on *DDX39B* mutations [42]. Polymorphisms in *DDX39B (BAT1)* have been described in several diseases with inflammatory profiles, including neuropathy, myasthenia gravis, allergies, Alzheimer disease, myocardial infarction, hepatitis, rheumatoid arthritis, Chagas disease, among others [25,43-48]. In this study it was found that the G allele of *DDX39B*-22C > G may be a resistance factor to malaria and the C allele a risk factor for disease complications. It has been reported that *DDX39B* promoter polymorphisms alter the binding of transcription factors (YY1 and Oct1) and may affect the transcription of this gene, and the sequences with -22G and -348 T alleles were expressed more efficiently than sequences containing -22C and-348C alleles [6]. An in vitro study found that BAT1 appeared to decrease the expression of TNF and IL-6 [5]; thus the G allele of *DDX39B*-22C > G, which enhances the expression of BAT1, may be protective against complicated malaria by decreasing the expression of proinflammatory cytokines.

Although the polymorphisms described in this study are not directly associated with the clinical manifestations of malaria, they can indirectly influence disease by altering the levels of inflammatory mediators involved in disease immunopathology. High plasma levels of TNF are related to the pathogenesis of signs associated with malaria, such as fever, and severe forms of infection, such as cerebral malaria and severe anemia [49]. Our study found that participants heterozygous (AG) for *TNF*-308G > A had higher plasma concentrations of TNF than homozygous participants with the wild-type (GG) polymorphism. The A allele of this SNP has been associated with increased production of TNF in several studies, and was often associated with the HLA-A1-B8-DR3 haplotype of the MHC region [50-53]. In this study results did not clearly demonstrate increased levels of TNF in participants homozygous for the A mutation, probably because of the small number of participants with this genotype who were recruited for the study. Furthermore, participants heterozygous (CT) for *DDX39B*-348C > T had higher plasma TNF than homozygous participants with the wild-type (CC) polymorphism, suggesting that an additional genetic mechanism appears to be associated with increased levels of this cytokine, and consequently the clinical outcome of malaria infection. CRP is an acute-phase inflammatory protein, and this study findings indicate that participants homozygous (CC) for *DDX39B*-22 (G > C) had increased levels of CRP, supporting an association of the C allele with risk of complicated malaria.

A single-point mutation is often not sufficient for predicting the susceptibility or resistance of individuals to malaria [4]. Another approach to investigating the differences in response to malaria infection is haplotype analysis of mutant alleles. *DDX39B* is situated in the central region of MHC on the short arm of human chromosome 6 and is approximately 150 kb from the *TNF* gene. The *NFKBIL1* gene, which encodes the inhibitor of κB-like protein (IκBL), a protein of unknown function, is situated between *DDX39B* and *TNF* [54]. Genetic variations in *NFKBIL1* are associated with susceptibility to inflammatory conditions such as periodontitis, chronic thromboembolic pulmonary hypertension, rheumatoid arthritis, and malaria [54-58]. Although *DDX39B* and *TNF* genes are near each other on the same chromosome and appear to influence the transcription of its gene products [5], this study did not identify a haplotype with *DDX39B* (22C > G and 348C > T) and *TNF*-308G > A polymorphisms that increased the risk of clincal vivax malaria. However, many haplotypes appear to markedly increase TNF levels, indirectly contributing to malaria susceptibility. It is noteworthy that conclusive findings from *DDX39B* and *TNF* haplotype analysis are limited by the considerable distance between these genes (approximately 150 kb). Similarly, it is reported here an analysis of susceptibility to clinical manifestations of malaria as a result of genotype combinations found that CG/CC/GG/GG and GG/CT/GG/GG, corresponding to the respective polymorphisms *DDX39B*-22/*DDX39B*-348/*TNF*-308/*IL6*-176, were associated with decreased and increased risk, respectively, of developing clinical manifestations of *P. vivax* infection. Intriguingly, *TNF*-308 and *IL6*-176 genotypes were wild-type homozygotes (GG for both) among the combinations, and changes were related to *DDX39B* genotypes. The substitution of *DDX39B* genotypes (−22 and −348) in the combinations completely changed the risk of developing manifestations of malaria, from susceptibility to resistance to illness from vivax malaria infections. These results lend support to the role of *DDX39B* as a regulatory gene that can alter transcription factors and inflammatory cytokines and influence the clinical outcome of inflammatory diseases [5,25]. Moreover, study participants with the genotype combination described here that was associated with resistance against manifestations of *P. vivax* infection (CG/CC/GG/GG) also had lower levels of proinflammatory TNF and IL-6, suggesting that *DDX39B* confers protection against malaria pathogenesis by reducing the inflammatory response.

This study was limited because of a small numbers of participants, which may have reduced its ability to detect significant differences between study groups. Indeed, the detection of a small difference (effect size) between groups at a significance level of 0.05 ideally would require at least 1,194 study participants. Therefore, our study results require validation in larger studies.

Conclusion

Genetic alterations in the immune response against vivax malaria may predispose individuals to disease complications or protect them from clinical disease. The findings of this study provide support that the C allele of *DDX39B*-22C > G is a risk factor of complicated vivax malaria, and different haplotypes (including *DDX39B* and *TNF* polymorphisms) may influence disease outcomes by altering plasma levels of TNF. Moreover, the results suggest that combinations of genotypes (including *IL6*-176G > C) are associated with a decreased or increased risk of developing clinical manifestations of malaria and may also influence plasma TNF and IL-6 levels. Further prospective studies should be able to determine if these genetic determinants are critical for protection against the development of clinical *P. vivax* infection, and also identify individuals who are at risk of developing more complicated forms of malaria.

Abbreviations

DDX39B: DEAD [Asp-Glu-Ala-Asp] box polypeptide 39B; BAT1: HLA-B associated transcript 1; RNA: Ribonucleic acid; TNF: Tumor necrosis factor; IL-6: Interleukin 6; SNP: Single nucleotide polymorphism; CXCL10: C-X-C motif chemokine 10; CRP: C-reactive protein; FUNASA: Brazilian National Foundation of Health; PCR: Polymerase chain reaction; FIOCRUZ: Oswaldo Cruz Foundation; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HIV: Human immunodeficiency virus; FMT-HVD: Fundação de Medicina Tropical Dr Heitor Vieira Dourado; DNA: Deoxyribonucleic acid; OR: Odds ratio; CI: Confidence interval; HWE: Hardy-Weinberg equilibrium; IQR: Interquartile range; MHC: Major histocompatibility complex; IκBL: Inhibitor of κB-like protein.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VRRM performed the experiments, analyzed the data, and wrote the manuscript together with BBA and MBN. LCLS and GCG helped perform the experiments. BBA performed the field study and sampling in Rondonia and performed the plasma measurements and wrote the manuscript. BMLM was responsible for the field study and sampling in Manaus. MVGL supervised the clinical study and sampling in Manaus. MBN conceptualized the study, supervised the clinical study in Rondonia, and helped with data interpretation and the writing of the manuscript. All authors have read and approved the final version of the manuscript.

Acknowledgements

The authors thank João Gambati (FUNASA, Brazil), Luis Marcelo Camargo (USP, Brazil) and our team at FIOCRUZ-Bahia, Brazil: Jorge Tolentino, Adorielze Leite, Nataly Alexandrino and Andrezza Karíny, who help in logistics of the field study and experiments.

This work was supported by FIOCRUZ and FMT-HVD. MBN and MSG are senior investigators from CNPq. MVGL is scientific director at FMT-HVD. VRRM, LCLS, GCG, and BMLM are supported by CNPq. The work of BBA is supported by the intramural research programme of the National Institute of Allergy and Infectious Diseases, National Institute of Health. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author details

[1]Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Brazil. [2]Faculdade de Medicina, Universidade Federal da Bahia, Salvador, Brazil. [3]Fundação de Medicina Tropical Dr Heitor Vieira Dourado, Manaus, Brazil. [4]Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA. [5]Instituto de Investigação em Imunologia, Instituto Nacional de Ciência e Tecnologia, São Paulo, Brazil.

Received: 16 April 2014 Accepted: 14 July 2014
Published: 19 July 2014

References

1. WHO: *World Malaria Report 2013*. Geneva: World Health Organization; 2013.
2. Andrade BB, Barral-Netto M: **Biomarkers for susceptibility to infection and disease severity in human malaria.** *Mem Inst Oswaldo Cruz* 2011, **106**(Suppl):70–78.
3. Mackinnon MJ, Mwangi TW, Snow RW, Marsh K, Williams TN: **Heritability of malaria in Africa.** *PLoS Med* 2005, **2**:e340.
4. De Mendonça VRR, Gonçalves MS, Barral-Netto M: **The host genetic diversity in malaria infection.** *J Trop Med* 2012, **2012**:940616.
5. Allcock RJ, Williams JH, Price P: **The central MHC gene, BAT1, may encode a protein that down-regulates cytokine production.** *Genes Cells* 2001, **6**:487–494.
6. Price P, Wong AM-L, Williamson D, Voon D, Baltic S, Allcock RJN, Boodhoo A, Christiansen FT: **Polymorphisms at positions −22 and −348 in the promoter of the BAT1 gene affect transcription and the binding of nuclear factors.** *Hum Mol Genet* 2004, **13**:967–974.
7. Andrade BB, Reis-Filho A, Souza-Neto SM, Raffaele-Netto I, Camargo LMA, Barral A, Barral-Netto M: **Plasma superoxide dismutase-1 as a surrogate marker of vivax malaria severity.** *PLoS Negl Trop Dis* 2010, **4**:e650.
8. Sohail M, Kaul A, Bali P, Raziuddin M, Singh MP, Singh OP, Dash AP, Adak T: **Alleles -308A and -1031C in the TNF-alpha gene promoter do not increase the risk but associated with circulating levels of TNF-alpha and clinical features of vivax malaria in Indian patients.** *Mol Immunol* 2008, **45**:1682–1692.
9. Aubouy A, Deloron P, Migot-Nabias F: **Plasma and in vitro levels of cytokines during and after a *Plasmodium falciparum* malaria attack in Gabon.** *Acta Trop* 2002, **83**:195–203.
10. Böttger E, Grangeiro de Carvalho E, Meese S, Kun JFJ, Esen M: **Expression of interleukin-6 family receptors in NK92 cells is regulated by cytokines and not through direct interaction with *Plasmodium falciparum*-infected erythrocytes.** *J Interferon Cytokine Res* 2013, **33**:65–71.
11. Gonçalves RM, Scopel KKG, Bastos MS, Ferreira MU: **Cytokine balance in human malaria: does *Plasmodium vivax* elicit more inflammatory responses than *Plasmodium falciparum*?** *PLoS One* 2012, **7**:e44394.
12. Lyke KE, Burges R, Cissoko Y, Sangare L, Dao M, Diarra I, Kone A, Harley R, Plowe CV, Doumbo OK, Sztein MB: **Serum levels of the proinflammatory cytokines interleukin-1 beta (IL-1beta), IL-6, IL-8, IL-10, tumor necrosis factor alpha, and IL-12(p70) in Malian children with severe *Plasmodium falciparum* malaria and matched uncomplicated malaria or healthy controls.** *Infect Immun* 2004, **72**:5630–5637.
13. Abraham LJ, Kroeger KM: **Impact of the −308 TNF promoter polymorphism on the transcriptional regulation of the TNF gene: relevance to disease.** *J Leukoc Biol* 1999, **66**:562–566.
14. Kilpinen S, Hulkkonen J, Wang XY, Hurme M: **The promoter polymorphism of the interleukin-6 gene regulates interleukin-6 production in neonates but not in adults.** *Eur Cytokine Netw* 2001, **12**:62–68.
15. Brull DJ, Montgomery HE, Sanders J, Dhamrait S, Luong L, Rumley A, Lowe GD, Humphries SE: **Interleukin-6 gene -174 g > c and -572 g > c promoter polymorphisms are strong predictors of plasma interleukin-6 levels after coronary artery bypass surgery.** *Arterioscler Thromb Vasc Biol* 2001, **21**:1458–1463.
16. Andrade BB, Rocha BC, Reis-Filho A, Camargo LMA, Tadei WP, Moreira LA, Barral A, Barral-Netto M: **Anti-*Anopheles darlingi* saliva antibodies as marker of *Plasmodium vivax* infection and clinical immunity in the Brazilian Amazon.** *Malar J* 2009, **8**:121.
17. Andrade BB, Reis-Filho A, Barros AM, Souza-Neto SM, Nogueira LL, Fukutani KF, Camargo EP, Camargo LMA, Barral A, Duarte A, Barral-Netto M: **Towards a precise test for malaria diagnosis in the Brazilian Amazon: comparison among field microscopy, a rapid diagnostic test, nested PCR, and a computational expert system based on artificial neural networks.** *Malar J* 2010, **9**:117.
18. Andrade BB, Reis-Filho A, Souza-Neto SM, Clarêncio J, Camargo LMA, Barral A, Barral-Netto M: **Severe *Plasmodium vivax* malaria exhibits marked inflammatory imbalance.** *Malar J* 2010, **9**:13.
19. Andrade BB, Araújo-Santos T, Luz NF, Khouri R, Bozza MT, Camargo LMA, Barral A, Borges VM, Barral-Netto M: **Heme impairs prostaglandin E2 and TGF-beta production by human mononuclear cells via Cu/Zn superoxide**

dismutase: insight into the pathogenesis of severe malaria. *J Immunol* 2010, **185**:1196–1204.
20. Andrade BB, Santos CJN, Camargo LM, Souza-Neto SM, Reis-Filho A, Clarêncio J, Mendonça VRR, Luz NF, Camargo EP, Barral A, Silva AAM, Barral-Netto M: **Hepatitis B infection is associated with asymptomatic malaria in the Brazilian Amazon.** *PLoS One* 2011, **6**:e19841.
21. Mendonça VRR, Luz NF, Santos NJG, Borges VM, Gonçalves MS, Andrade BB, Barral-Netto M: **Association between the haptoglobin and heme oxygenase 1 genetic profiles and soluble CD163 in susceptibility to and severity of human malaria.** *Infect Immun* 2012, **80**:1445–1454.
22. Gozzelino R, Andrade BB, Larsen R, Luz NF, Vanoaica L, Seixas E, Coutinho A, Cardoso S, Rebelo S, Poli M, Barral-Netto M, Darshan D, Kühn LC, Soares MP: **Metabolic Adaptation to tissue iron overload confers tolerance to malaria.** *Cell Host Microbe* 2012, **12**:693–704.
23. Mendonça VRR, Queiroz ATL, Lopes FM, Andrade BB, Barral-Netto M: **Networking the host immune response in *Plasmodium vivax* malaria.** *Malar J* 2013, **12**:69.
24. WHO: *Guidelines for the treatment of malaria. Second edition*. Geneva: World Health Organization; 2010.
25. Ramasawmy R, Cunha-Neto E, Faé KC, Müller NG, Cavalcanti VL, Drigo SA, Ianni B, Mady C, Kalil J, Goldberg AC: **BAT1, a putative anti-inflammatory gene, is associated with chronic Chagas cardiomyopathy.** *J Infect Dis* 2006, **193**:1394–1399.
26. Yalçın S, Kayaalti Z, Söylemezoğlu T: **Role of interleukin-6–174 G/C promoter polymorphism in trace metal levels of autopsy kidney and liver tissues.** *Int J Hyg Environ Health* 2011, **214**:219–224.
27. Wang T-N, Chen W-Y, Wang T-H, Chen C-J, Huang L-Y, Ko Y-C: **Gene-gene synergistic effect on atopic asthma: tumour necrosis factor-alpha-308 and lymphotoxin-alpha-NcoI in Taiwan's children.** *Clin Exp Allergy* 2004, **34**:184–188.
28. Anand AC, Puri P: **Jaundice in malaria.** *J Gastroenterol Hepatol* 2005, **20**:1322–1332.
29. Fabbri C, de Cássia Mascarenhas-Netto R, Lalwani P, Melo GC, Magalhães BML, Alexandre MAA, Lacerda MVG, Lima ES: **Lipid peroxidation and antioxidant enzymes activity in *Plasmodium vivax* malaria patients evolving with cholestatic jaundice.** *Malar J* 2013, **12**:315.
30. Lyke KE, Fernández-Viña MA, Cao K, Hollenbach J, Coulibaly D, Kone AK, Guindo A, Burdett LA, Hartzman RJ, Wahl AR, Hildebrand WH, Doumbo OK, Plowe CV, Sztein MB: **Association of HLA alleles with *Plasmodium falciparum* severity in Malian children.** *Tissue Antigens* 2011, **77**:562–571.
31. Flori L, Sawadogo S, Esnault C, Delahaye NF, Fumoux F, Rihet P: **Linkage of mild malaria to the major histocompatibility complex in families living in Burkina Faso.** *Hum Mol Genet* 2003, **12**:375–378.
32. Diakite M, Clark TG, Auburn S, Campino S, Fry AE, Green A, Morris AP, Richardson A, Jallow M, Sisay-Joof F, Pinder M, Kwiatkowski DP, Rockett KA: **A genetic association study in the Gambia using tagging polymorphisms in the major histocompatibility complex class III region implicates a HLA-B associated transcript 2 polymorphism in severe malaria susceptibility.** *Hum Genet* 2009, **125**:105–109.
33. Day NP, Hien TT, Schollaardt T, Loc PP, Chuong LV, Chau TT, Mai NT, Phu NH, Sinh DX, White NJ, Ho M: **The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria.** *J Infect Dis* 1999, **180**:1288–1297.
34. Kern P, Hemmer CJ, Van Damme J, Gruss HJ, Dietrich M: **Elevated tumor necrosis factor alpha and interleukin-6 serum levels as markers for complicated *Plasmodium falciparum* malaria.** *Am J Med* 1989, **87**:139–143.
35. Israelsson E, Maiga B, Kearsley S, Dolo A, Homann MV, Doumbo OK, Troye-Blomberg M, Tornvall P, Berzins K: **Cytokine gene haplotypes with a potential effect on susceptibility to malaria in sympatric ethnic groups in Mali.** *Infect Genet Evol* 2011, **11**:1608–1615.
36. McGuire W, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D: **Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria.** *Nature* 1994, **371**:508–510.
37. Clark TG, Diakite M, Auburn S, Campino S, Fry AE, Green A, Richardson A, Small K, Teo YY, Wilson J, Jallow M, Sisay-Joof F, Pinder M, Griffiths MJ, Peshu N, Williams TN, Marsh K, Molyneux ME, Taylor TE, Rockett KA, Kwiatkowski DP: **Tumor necrosis factor and lymphotoxin-alpha polymorphisms and severe malaria in African populations.** *J Infect Dis* 2009, **199**:569–575.
38. Meyer CG, May J, Luty AJ, Lell B, Kremsner PG: **TNFalpha-308A associated with shorter intervals of *Plasmodium falciparum* reinfections.** *Tissue Antigens* 2002, **59**:287–292.
39. Wattavidanage J, Carter R, Perera KL, Munasingha A, Bandara S, McGuinness D, Wickramasinghe AR, Alles HK, Mendis KN, Premawansa S: **TNFalpha*2 marks high risk of severe disease during *Plasmodium falciparum* malaria and other infections in Sri Lankans.** *Clin Exp Immunol* 1999, **115**:350–355.
40. Sortica VA, Cunha MG, Ohnishi MDO, Souza JM, Ribeiro-Dos-Santos AKC, Santos NPC, Callegari-Jacques SM, Santos SEB, Hutz MH: **IL1B, IL4R, IL12RB1 and TNF gene polymorphisms are associated with *Plasmodium vivax* malaria in Brazil.** *Malar J* 2012, **11**:409.
41. Knight JC, Keating BJ, Kwiatkowski DP: **Allele-specific repression of lymphotoxin-alpha by activated B cell factor-1.** *Nat Genet* 2004, **36**:394–399.
42. Flori L, Delahaye NF, Iraqi FA, Hernandez-Valladares M, Fumoux F, Rihet P: **TNF as a malaria candidate gene: polymorphism-screening and family-based association analysis of mild malaria attack and parasitemia in Burkina Faso.** *Genes Immun* 2005, **6**:472–480.
43. Cherry CL, Rosenow A, Affandi JS, McArthur JC, Wesselingh SL, Price P: **Cytokine genotype suggests a role for inflammation in nucleoside analog-associated sensory neuropathy (NRTI-SN) and predicts an individual's NRTI-SN risk.** *AIDS Res Hum Retroviruses* 2008, **24**:117–123.
44. Degli-Esposti MA, Leelayuwat C, Dawkins RL: **Ancestral haplotypes carry haplotypic and haplospecific polymorphisms of BAT1: possible relevance to autoimmune disease.** *Eur J Immunogenet* 1992, **19**:121–127.
45. Gnjec A, D'Costa KJ, Laws SM, Hedley R, Balakrishnan K, Taddei K, Martins G, Paton A, Verdile G, Gandy SE, Broe GA, Brooks WS, Bennett H, Piguet O, Price P, Mildossy J, Hallmayer J, McGeer PL, Martins RN: **Association of alleles carried at TNFA −850 and BAT1–22 with Alzheimer's disease.** *J Neuroinflammation* 2008, **5**:36.
46. Koch W, Hoppmann P, Michou E, Jung V, Pfeufer A, Mueller JC, Gieger C, Wichmann H-E, Meitinger T, Schömig A, Kastrati A: **Association of variants in the BAT1-NFKBIL1-LTA genomic region with protection against myocardial infarction in Europeans.** *Hum Mol Genet* 2007, **16**:1821–1827.
47. Oliveira LC, Porta G, Marin MLC, Bittencourt PL, Kalil J, Goldberg AC: **Autoimmune hepatitis, HLA and extended haplotypes.** *Autoimmun Rev* 2011, **10**:189–193.
48. Quiñones-Lombraña A, Lopez-Soto A, Ballina-Garcia FJ, Alperi-López M, Queiro-Silva R, Lopez-Vazquez A, Lopez-Larrea C, Gonzalez S: **BAT1 promoter polymorphism is associated with rheumatoid arthritis susceptibility.** *J Rheumatol* 2008, **35**:741–744.
49. Karunaweera ND, Grau GE, Gamage P, Carter R, Mendis KN: **Dynamics of fever and serum levels of tumor necrosis factor are closely associated during clinical paroxysms in *Plasmodium vivax* malaria.** *Proc Natl Acad Sci U S A* 1992, **89**:3200–3203.
50. Wilson AG, de Vries N, Pociot F, di Giovine FS, van der Putte LB, Duff GW: **An allelic polymorphism within the human tumor necrosis factor alpha promoter region is strongly associated with HLA A1, B8, and DR3 alleles.** *J Exp Med* 1993, **177**:557–560.
51. Jacob CO, Fronek Z, Lewis GD, Koo M, Hansen JA, McDevitt HO: **Heritable major histocompatibility complex class II-associated differences in production of tumor necrosis factor alpha: relevance to genetic predisposition to systemic lupus erythematosus.** *Proc Natl Acad Sci U S A* 1990, **87**:1233–1237.
52. Candore G, Cigna D, Gervasi F, Colucci AT, Modica MA, Caruso C: **In vitro cytokine production by HLA-B8, DR3 positive subjects.** *Autoimmunity* 1994, **18**:121–132.
53. Bouma G, Crusius JB, Oudkerk Pool M, Kolkman JJ, von Blomberg BM, Kostense PJ, Giphart MJ, Schreuder GM, Meuwissen SG, Peña AS: **Secretion of tumour necrosis factor alpha and lymphotoxin alpha in relation to polymorphisms in the TNF genes and HLA-DR alleles. Relevance for inflammatory bowel disease.** *Scand J Immunol* 1996, **43**:456–463.
54. An J, Nakajima T, Shibata H, Arimura T, Yasunami M, Kimura A: **A novel link of HLA locus to the regulation of immunity and infection: NFKBIL1 regulates alternative splicing of human immune-related genes and influenza virus M gene.** *J Autoimmun* 2013, **47**:25–33.
55. Atkinson SH, Rockett KA, Morgan G, Bejon PA, Sirugo G, O'Connell MA, Hanchard N, Kwiatkowski DP, Prentice AM: **Tumor necrosis factor SNP haplotypes are associated with iron deficiency anemia in West African children.** *Blood* 2008, **112**:4276–4283.
56. Kominami S, Tanabe N, Ota M, Naruse TK, Katsuyama Y, Nakanishi N, Tomoike H, Sakuma M, Shirato K, Takahashi M, Shibata H, Yasunami M, Chen Z, Kasahara Y, Tatsumi K, Kuriyama T, Kimura A: **HLA-DPB1 and NFKBIL1 may confer the susceptibility to chronic thromboembolic pulmonary**

hypertension in the absence of deep vein thrombosis. *J Hum Genet* 2009, **54**:108–114.
57. Chiba T, Matsuzaka Y, Warita T, Sugoh T, Miyashita K, Tajima A, Nakamura M, Inoko H, Sato T, Kimura M: **NFKBIL1 confers resistance to experimental autoimmune arthritis through the regulation of dendritic cell functions.** *Scand J Immunol* 2011, **73**:478–485.
58. Kallio KAE, Marchesani M, Vlachopoulou E, Mäntylä P, Paju S, Buhlin K, Suominen AL, Contreras J, Knuuttila M, Hernandez M, Huumonen S, Nieminen MS, Perola M, Sinisalo J, Lokki M-L, Pussinen PJ: **Genetic variation on the BAT1-NFKBIL1-LTA region of major histocompatibility complex class III associates with periodontitis.** *Infect Immun* 2014, **82**:1939–1948.

doi:10.1186/1475-2875-13-278
Cite this article as: Mendonça *et al.*: *DDX39B* (*BAT1*), *TNF* and *IL6* gene polymorphisms and association with clinical outcomes of patients with *Plasmodium vivax* malaria. *Malaria Journal* 2014 **13**:278.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

BioMed Central

5.5 MANUSCRITO V

Associations between Hepcidin and Immune Response in Individuals with Hyperbilirubinaemia and Severe Malaria due to *Plasmodium vivax* Infection.

Este manuscrito investiga as associações entre os níveis plasmáticos da hepcidina e de citocinas/quimiocinas nos grupos de malária grave, malária com hiperbilirrubinemia isolada e malária moderada por *P. vivax*.

Resumo dos resultados: Os níveis elevados de IL-2 e IL-13 foram associados com malária grave quando comparados com o grupo de hiperbilirrubinemia, e não foram observadas diferenças na expressão dos marcadores imunes entre os indivíduos com malária modera e malária com hiperbilirrubinemia. Contudo, a hepcidina estava elevada em indivíduos com malária grave ou com hiperbilirrubinemia, e valores de cut-off deste hormônio conseguiram diferenciar estes grupos quando comparados com o grupo de malária moderada. IFN-γ e TNF tiveram o número relativo maior de interações na rede dos participantes com malária grave. Além disso, a hepcidina foi positivamente correlacionada com IL-6, IL-10 e parasitemia no grupo de malária moderada e com IFN-γ nos indivíduos graves.

Este trabalho foi publicado no periódico internacional *Malaria Journal.*

Mendonça et al. Malar J (2015) 14:407
DOI 10.1186/s12936-015-0930-x

MALARIA JOURNAL

RESEARCH Open Access

Associations between hepcidin and immune response in individuals with hyperbilirubinaemia and severe malaria due to *Plasmodium vivax* infection

Vitor R. R. Mendonça[1,2], Ligia C. L. Souza[1,2], Gabriela C. Garcia[1,2], Belisa M. L. Magalhães[3,4], Marilda S. Gonçalves[1], Marcus V. G. Lacerda[3,4] and Manoel Barral-Netto[1,2,5*]

Abstract

Background: Hyperbilirubinaemia (bilirubin >51.3 μmol/L) alone is not indicative of severe malaria, and the immune response underlying hyperbilirubinaemia remains largely unexplored. Liver damage associated with hyperbilirubinaemia may alter the expression of hepcidin, which regulates systemic iron by degrading ferroportin. For this study, the association between hepcidin and the levels of cytokines and chemokines in the serum of individuals with mild and severe vivax malaria and subjects with malaria with isolated hyperbilirubinaemia was evaluated.

Methods: Cytokines/chemokines and hepcidin were measured in individuals with mild (n = 72) and severe (n = 17) vivax malaria, as well as in the serum of subjects with vivax malaria with isolated hyperbilirubinaemia (n = 14) from the Brazilian Amazon between 2009 and 2013 by multiplex assay and ELISA, respectively. The polymorphism 744 G > T in the *ferroportin* gene was identified by restriction fragment-length polymorphism analysis and the restriction enzyme *Pvu*II.

Results: The polymorphism at position 744 G > T in the *ferroportin* gene was typed and no differences in the distributions of genotypes or alleles were observed between the study groups. Subjects with severe malaria had higher levels of interleukin (IL)-2 and IL-13 than subjects with hyperbilirubinaemia. No differences in the expression of immune markers were observed between subjects with mild malaria and those with hyperbilirubinaemia. However, hepcidin levels were higher in individuals with severe malaria and hyperbilirubinaemia than those with mild malaria ($p = 0.0002$ and $p = 0.0004$, respectively) and cut-off values of hepcidin differentiated these groups from subjects with mild malaria. Hepcidin was positively associated with IL-6 and IL-10 levels and with parasitaemia in subjects with mild malaria and with IFN-γ in subjects with severe malaria.

Conclusions: Malaria in the presence of hyperbilirubinaemia produces a less robust inflammatory response compared to severe cases of malaria. Hepcidin levels are positively associated with immune markers in vivax malaria outcomes.

Keywords: Malaria, *Plasmodium vivax*, Immune response, Hepcidin, Hyperbilirubinemia

Background

Malaria is a major health problem worldwide, and in 2013 it was estimated to cause 584,000 deaths [1]. *Plasmodium vivax* is the most widespread malaria parasite and is responsible for the majority of malaria cases in Southeast Asia and South America [1]. Clinical outcomes from *Plasmodium* infections can range from severe or mild diseases to asymptomatic parasite carriers. The balance and interactions between anti- and pro-inflammatory cytokines play an important role in vivax malaria manifestations [2]. Further, genetic alterations in genes related

*Correspondence: mbarral@bahia.fiocruz.br
[5] Instituto de Investigação em Imunologia, Instituto Nacional de Ciência e Tecnologia, São Paulo, Brazil
Full list of author information is available at the end of the article

BioMed Central

© 2015 Mendonça et al. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

to immune response have been associated with clinical outcomes [3, 4].

Hyperbilirubinaemia, which is also known as jaundice, is often associated with malaria infection. It occurs as a consequence of the intravascular haemolysis, disseminated intravascular coagulation and hepatocellular dysfunction [5]. However, recently, jaundice (serum total bilirubin >3 mg/dL) is no longer considered a single marker of malaria severity and the presence of hyperbilirubinaemia alone is not associated with a worse prognosis or a higher fatality rate in malaria [5–8]. Nevertheless, hyperbilirubinaemia is a common complication associated with severe malaria syndromes and concomitant jaundice can indicate more severe illness [9–12].

The rupture of red blood cells in the blood stream during malaria infection is associated with an increase in indirect bilirubin, but the primary schizogony of the malarial parasite also leads to the rupture of infected hepatocytes and elevates direct bilirubin levels. Both of these factors contribute to hyperbilirubinaemia and clinical jaundice [12–16]. In this context, the hepatocellular damage observed in individuals with malaria and hyperbilirubinaemia may alter the hepatocytic expression of hepcidin, which regulates systemic iron homeostasis by degrading ferroportin, the only known human iron cell exporter [17]. The degradation of ferroportin leads to the inhibition of intestinal absorption of dietary iron and accumulation of iron in macrophages leading to low iron availability [17].

The immune response underlying malaria-related jaundice, which is defined by high productions of interleukin (IL)-6, IL-10 and interferon (IFN)-γ, may influence hepcidin levels in hepatocytes and peripheral blood mononuclear cells [18, 19]. Portugal et al. described that increased hepcidin levels during a *Plasmodium* blood-stage infection inhibited subsequent liver infection in a rodent model [20]. Further, hepcidin levels are regulated by inflammation, hypoxia, iron status, and IL-6 production [21–27]. Hepcidin levels are increased in children during acute *P. falciparum* uncomplicated malaria [23] as well asymptomatic malaria caused by *P. falciparum* or *P. vivax* [24]; however, children with severe falciparum malaria demonstrated to have very low levels of this hormone [25–27]. Recently, hepcidin levels were demonstrated to be the best predictor of iron absorption in children under competing conditions, such as anaemia, iron deficiency and infection [28]. This suggests a potential utility for hepcidin in managing iron supplementation programmes at the time of malaria infection because of the inhibitory effect of hepcidin on the absorption of oral iron.

Noteworthy, no previous studies involving hepcidin in adults with symptomatic vivax malaria and in adults with severe malaria have been done so far. Hepcidin has been found to be low in children with severe malaria [25–27], but adults with severe malaria have not been evaluated. In the present study, it was studied the associations between hepcidin and the levels of cytokines and chemokines in the serum of adults with severe and mild vivax malaria, as well as in subjects with vivax malaria with isolated hyperbilirubinaemia. The results herein indicate that individuals with hyperbilirubinaemia alone exhibit an inflammatory response similar to subjects with mild infections without hyperbilirubinaemia. Of equal importance is the finding that subjects with malaria and hyperbilirubinaemia and subjects with severe malaria have elevated levels of hepcidin; cut-off values of hepcidin can differentiate these groups of subjects from those with mild malaria. Further, hepcidin levels are associated with immune responses in the different *P. vivax* infection study groups.

Methods

Study design and participants

Individuals with an acute febrile syndrome (age range, 4 months to 76 years; median age 36 years) who sought care at the reference hospital at the Fundação de Medicina Tropical Dr Heitor Vieira Dourado (FMT-HVD), Amazonas, Brazil, were recruited between 2009 and 2013. Subjects were tested for malaria by thick blood smear and those with *P. vivax* infections confirmed by PCR were invited to participate in this study. All patients with vivax malaria who developed haemolysis due to primaquine treatment (i.e., patients using primaquine who experienced haemoglobin <10 g/dL and reticulocyte count >1.5 % or an indirect increase in bilirubin levels after using primaquine) were excluded. Patients with microscopic or molecular diagnosis of malaria caused by *Plasmodium falciparum* or *P. vivax* and *P. falciparum* co-infection and patients with a serologic diagnosis of viral hepatitis (A, B, C, or D), HIV, or leptospirosis were also excluded. Participants were categorized into three groups based on the severity of infection: mild malaria (n = 72; without hyperbilirubinaemia), malaria with hyperbilirubinaemia (n = 14) and severe malaria (n = 17). Severe vivax malaria was defined according to the criteria for severe malaria established by the World Health Organization (WHO); malaria with hyperbilirubinaemia was defined by a serum total bilirubin level >51.3 μmol/L with no other criteria for severe malaria [6]. Details of subjects with severe malaria and hyperbilirubinaemia can be found in Mendonça et al. [3].

Ethics statement

This study was approved by the Ethics Committee of the FMT-HVD (protocol number: 2009/15243) and all

to immune response have been associated with clinical outcomes [3, 4].

Hyperbilirubinaemia, which is also known as jaundice, is often associated with malaria infection. It occurs as a consequence of the intravascular haemolysis, disseminated intravascular coagulation and hepatocellular dysfunction [5]. However, recently, jaundice (serum total bilirubin >3 mg/dL) is no longer considered a single marker of malaria severity and the presence of hyperbilirubinaemia alone is not associated with a worse prognosis or a higher fatality rate in malaria [5–8]. Nevertheless, hyperbilirubinaemia is a common complication associated with severe malaria syndromes and concomitant jaundice can indicate more severe illness [9–12].

The rupture of red blood cells in the blood stream during malaria infection is associated with an increase in indirect bilirubin, but the primary schizogony of the malarial parasite also leads to the rupture of infected hepatocytes and elevates direct bilirubin levels. Both of these factors contribute to hyperbilirubinaemia and clinical jaundice [12–16]. In this context, the hepatocellular damage observed in individuals with malaria and hyperbilirubinaemia may alter the hepatocytic expression of hepcidin, which regulates systemic iron homeostasis by degrading ferroportin, the only known human iron cell exporter [17]. The degradation of ferroportin leads to the inhibition of intestinal absorption of dietary iron and accumulation of iron in macrophages leading to low iron availability [17].

The immune response underlying malaria-related jaundice, which is defined by high productions of interleukin (IL)-6, IL-10 and interferon (IFN)-γ, may influence hepcidin levels in hepatocytes and peripheral blood mononuclear cells [18, 19]. Portugal et al. described that increased hepcidin levels during a *Plasmodium* blood-stage infection inhibited subsequent liver infection in a rodent model [20]. Further, hepcidin levels are regulated by inflammation, hypoxia, iron status, and IL-6 production [21–27]. Hepcidin levels are increased in children during acute *P. falciparum* uncomplicated malaria [23] as well asymptomatic malaria caused by *P. falciparum* or *P. vivax* [24]; however, children with severe falciparum malaria demonstrated to have very low levels of this hormone [25–27]. Recently, hepcidin levels were demonstrated to be the best predictor of iron absorption in children under competing conditions, such as anaemia, iron deficiency and infection [28]. This suggests a potential utility for hepcidin in managing iron supplementation programmes at the time of malaria infection because of the inhibitory effect of hepcidin on the absorption of oral iron.

Noteworthy, no previous studies involving hepcidin in adults with symptomatic vivax malaria and in adults with severe malaria have been done so far. Hepcidin has been found to be low in children with severe malaria [25–27], but adults with severe malaria have not been evaluated. In the present study, it was studied the associations between hepcidin and the levels of cytokines and chemokines in the serum of adults with severe and mild vivax malaria, as well as in subjects with vivax malaria with isolated hyperbilirubinaemia. The results herein indicate that individuals with hyperbilirubinaemia alone exhibit an inflammatory response similar to subjects with mild infections without hyperbilirubinaemia. Of equal importance is the finding that subjects with malaria and hyperbilirubinaemia and subjects with severe malaria have elevated levels of hepcidin; cut-off values of hepcidin can differentiate these groups of subjects from those with mild malaria. Further, hepcidin levels are associated with immune responses in the different *P. vivax* infection study groups.

Methods

Study design and participants

Individuals with an acute febrile syndrome (age range, 4 months to 76 years; median age 36 years) who sought care at the reference hospital at the Fundação de Medicina Tropical Dr Heitor Vieira Dourado (FMT-HVD), Amazonas, Brazil, were recruited between 2009 and 2013. Subjects were tested for malaria by thick blood smear and those with *P. vivax* infections confirmed by PCR were invited to participate in this study. All patients with vivax malaria who developed haemolysis due to primaquine treatment (i.e., patients using primaquine who experienced haemoglobin <10 g/dL and reticulocyte count >1.5 % or an indirect increase in bilirubin levels after using primaquine) were excluded. Patients with microscopic or molecular diagnosis of malaria caused by *Plasmodium falciparum* or *P. vivax* and *P. falciparum* co-infection and patients with a serologic diagnosis of viral hepatitis (A, B, C, or D), HIV, or leptospirosis were also excluded. Participants were categorized into three groups based on the severity of infection: mild malaria (n = 72; without hyperbilirubinaemia), malaria with hyperbilirubinaemia (n = 14) and severe malaria (n = 17). Severe vivax malaria was defined according to the criteria for severe malaria established by the World Health Organization (WHO); malaria with hyperbilirubinaemia was defined by a serum total bilirubin level >51.3 μmol/L with no other criteria for severe malaria [6]. Details of subjects with severe malaria and hyperbilirubinaemia can be found in Mendonça et al. [3].

Ethics statement

This study was approved by the Ethics Committee of the FMT-HVD (protocol number: 2009/15243) and all

subjects provided written informed consent. All clinical investigations were conducted according to the principles outlined in the Declaration of Helsinki.

Plasma measurements

Blood was obtained by venipuncture; heparinized plasma was separated and immediately used or stored at −70 °C. The following clinical markers were measured in fresh plasma samples at the clinical laboratory facility at the FMT-HVD (Manaus, Brazil): haemoglobin (HB), haematocrit (HT), platelets (PTL), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). Levels of hepcidin in thawed plasma were measured by enzyme-linked immunoassay (Assay Designs, Ann Arbor, MI, USA). Plasma levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17, IFN-γ, tumor necrosis factor (TNF), chemokines CCL2 and CCL4, granulocyte-colony stimulating factor (GCSF), and granulocyte–macrophage colony-stimulating factor (GMCSF) were measured using a multiplex assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA).

Genotyping

DNA was extracted from 200 μL of peripheral blood using a standard QIAGEN DNA blood mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. The polymorphism at position 744 G > T (rs11568350; G ancestral allele) in the *ferroportin* gene (*SLC40A1*) was typed by polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) analysis with the restriction enzyme *Pvu*II (New England Biolabs, Ipswich, MA, USA) according to the protocol previously published by Kasvosve et al. [29]. *SLC40A1* PCR products were size-separated by electrophoresis on 3 % agarose gel under non-denaturing conditions. PCR products were subsequently stained with ethidium bromide and visualized under a UV light.

Network analyses

Networks were generated from Spearman correlation matrices that contained values of each plasma marker measured in the samples; values were input into JMP 10.0 software (SAS, Cary, NC, USA). Each marker was selected as a target and the software performed a search within the other mediators for those that were correlated with the target and calculated a correlation matrix using Spearman rank tests. The features related to the selected target were linked, and the links shown in the networks represented statistically significant Spearman rank correlations ($P < 0.05$). To analyse the structure of the marker's network, the density of each network was calculated (range, 0–1). In the context of this study, the density was the ratio of the number of edges inferred in the network over the total number of possible edges between all pairs of nodes [2]. The network's figures were customized using the Ingenuity Systems Pathway Analysis software (Ingenuity Systems, Redwood City, CA, USA) and Adobe Illustrator (Adobe Systems Inc.).

Statistical analyses

Chi square or Fisher exact tests were applied to evaluate the associations between qualitative variables. The D'Agostino-Pearson omnibus normality test was used to test for Gaussian distribution of quantitative variables within the total sample. Variables that were not normally distributed were analysed with non-parametric tests. The Kruskal–Wallis test with Dunn's multiple comparison (when three groups were compared) and the Mann–Whitney test (when two groups were compared) were used to assess the differences among the clinical groups. Multinomial regression analyses adjusted for age and gender were performed to test associations between the plasma measurements (below or above the median values of the entire study population) and the different clinical conditions (mild malaria, hyperbilirubinaemia and severe malaria). The receiver operating characteristic (ROC) curves and C-statistics of markers were used to test the ability to distinguish between the different clinical groups. A hierarchical cluster analysis using Ward's method was performed to test whether a combination of different immune-related biomarkers could cluster the study groups separately. Hardy–Weinberg equilibrium (HWE) was assessed for the groups by comparing the observed number of different *SLC40A1* genotypes with those expected under HWE for the estimated allele frequency. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., USA), SPSS 19.0 (IBM, Armonk, NY, USA), and JMP 11.0 (SAS, Cary, NC, USA). A p value <0.05 was considered statically significant.

Results

Baseline characteristics and *SLC40A1* 744 G > T distribution of the study participants

Most of the study subjects were male: 77.78 % (n = 56) of the mild malaria group, 71.43 % (n = 10) of the malaria with hyperbilirubinaemia group and 52.94 % (n = 9) of the severe malaria group ($p = 0.0592$; Table 1). There were no significant differences in age among the study groups ($p = 0.1274$; Table 1). Parasitaemia levels were similar among the groups ($p = 0.2115$; Table 1). Subjects with severe malaria displayed lower levels of HB and HT than subjects with hyperbilirubinaemia and subjects with mild malaria ($p < 0.0001$ for both comparisons; Table 1). Individuals with hyperbilirubinaemia had the lowest PTL values ($p = 0.0003$) and the highest ALT values ($p = 0.0039$); AST levels did not differ among the groups ($p = 0.0686$; Table 1).

Table 1 Baseline characteristics, laboratory measurements, and distribution of *SLC40A1* 744 (G > T) polymorphism in the study participants

	Mild malaria (n = 72)	Malaria with hyperbilirubinaemia (n = 14)	Severe malaria (n = 17)	P-value
Male, n (%)	56 (77.78)	10 (71.43)	9 (52.94)	0.0592**
Age (year), median (IQR)	36.00 (27.00–45.50)	26.00 (20.25–36.25)	37.00 (10.50–44.50)	0.1274***
Parasitaemia (parasites/μL), median (IQR)	2673 (842–9313)	2,451 (247–11,655)	291 (0–29,184)	0.2115***
Laboratory measurements, median (IQR)				
Haemoglobin (g/dL)	13.20 (12.50–14.28)	11.75 (10.38–13.88)	7.10 (6.50–10.50)	<0.0001***
Haematocrit (%)	43.50 (40.65–46.18)	34.10 (30.20–41.45)	23.10 (19.75–31.90)	<0.0001***
Platelets (per mm3)	108,500 (71,250–132,250)	33,000 (18,000–51,250)	64,000 (33,500–202,000)	0.0003***
AST (IU/L)	67.50 (50.00–93.25)	65.00 (36.00–172.50)	34.00 (21.50–112.30)	0.0686***
ALT (IU/L)	34.00 (19.25–50.75)	104.50 (36.00–262.50)	41.50 (13.25–76.00)	0.0039***
SLC40A1 744 (G > T) n (%)				
GG	71 (98.61)	14 (100.00)	16 (94.12)	0.4108**
GT	1 (1.39)	0 (0.00)	1 (5.88)	
TT	0 (0.00)	0 (0.00)	0 (0.00)	
G allele	141 (99.30)	28 (100.00)	33 (97.06)	0.4199**
T allele	1 (0.70)	0 (0.00)	1 (2.94)	

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *IQR* interquartile range

** Categorized variables were compared using the Chi square test

*** Ordinal variables were compared using the Mann–Whitney test for two groups or the Kruskal–Wallis test with Dunn's multiple comparison for three or more groups

A very low frequency of the *ferroportin*-associated polymorphism (*SLC40A1* 744 G > T) was identified in the study subjects (Table 1). No individuals with the homozygous mutant-type (TT) genotype were identified; one (1.39 %) heterozygous individual (GT genotype) was identified in the mild malaria group and one (5.88 %) was identified in the severe malaria group (p = 0.4108; Table 1). No differences were observed in allele distributions among the study groups (p = 0.4199; Table 1). The frequencies of *SLC40A1* 744 G > T genotypes met the conditions for HWE in all categories of vivax malaria infection.

The use of hepcidin levels to differentiate subjects with severe malaria and hyperbilirubinaemia from subjects with mild malaria

Plasma levels of hepcidin were higher in individuals with severe malaria and hyperbilirubinaemia than subjects with mild malaria (p = 0.0002 and p = 0.0004, respectively; Fig. 1a). No difference in hepcidin levels was observed between the hyperbilirubinaemia and severe malaria groups (p = 0.9093). The ROC curves analysis and the C-statistics allowed for the calculation of the discriminatory power of hepcidin to distinguish subjects with hyperbilirubinaemia from those with severe malaria or mild malaria (Fig. 1b). Hepcidin levels >3.148 ng/mL offered a sensitivity of 85.71 %, a specificity of 76.39 % and an area under the curve (AUC) of 79.07 % to differentiate mild from severe malaria (Fig. 1b). Hepcidin levels >2.406 ng/mL offered a sensitivity of 82.35 %, a specificity of 70.83 % and an AUC of 78.02 % to differentiate mild malaria from malaria with hyperbilirubinaemia (Fig. 1b). Hepcidin median and cut-off values were established (Fig. 1b); an age- and gender-adjusted multinomial logistic regression analysis was conducted on the basis of these values to confirm the associations between high hepcidin levels and susceptibility to hyperbilirubinaemia and severe malaria (Fig. 1c). Hepcidin levels above the cut-off (3.148 ng/mL; OR 26.405, 95 % CI 4.789–145.596 ng/mL, p = 0.001) or median (1.810 ng/mL; OR 14.771, 95 % CI 2.563–85.120 ng/mL, p = 0.003) values were associated with hyperbilirubinaemia more frequently than mild malaria (Fig. 1c). Likewise, hepcidin levels above the cut-off (2.406 ng/mL; OR 12.861, 95 % CI 3.082–53.668 ng/mL, p = 0.001) or median (1.842 ng/mL; OR 14.332, 95 % CI 2.754–74.582 ng/mL, p = 0.002) values were associated with severe malaria more frequently than with mild malaria (Fig. 1c). Hepcidin concentrations of subjects in the smaller groups within the severe malaria group were not analysed due to the low number of individuals and the low power.

The expression of immune-related biomarkers in each of the study groups

A panel of 17 cytokines and chemokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17,

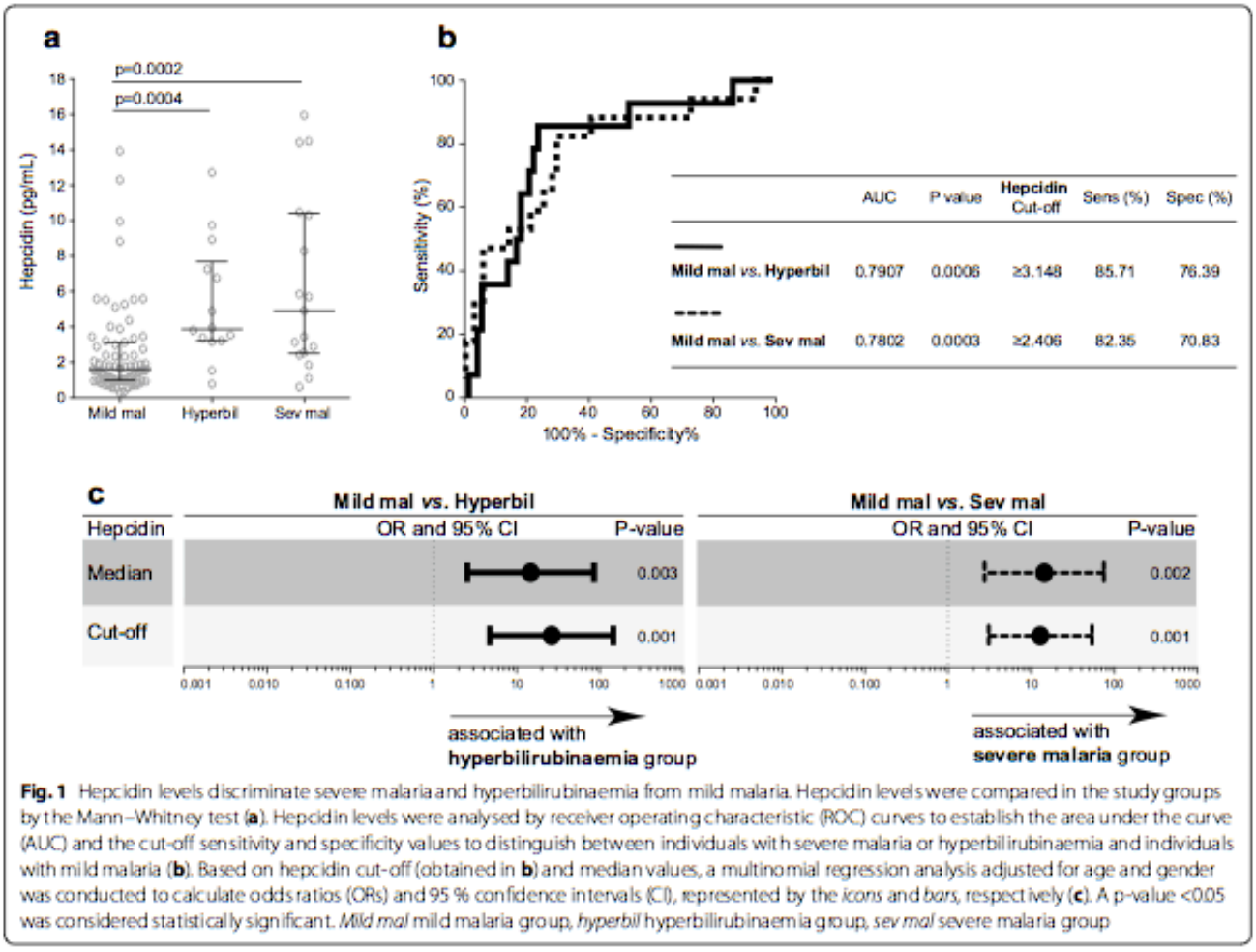


Fig. 1 Hepcidin levels discriminate severe malaria and hyperbilirubinaemia from mild malaria. Hepcidin levels were compared in the study groups by the Mann–Whitney test (**a**). Hepcidin levels were analysed by receiver operating characteristic (ROC) curves to establish the area under the curve (AUC) and the cut-off sensitivity and specificity values to distinguish between individuals with severe malaria or hyperbilirubinaemia and individuals with mild malaria (**b**). Based on hepcidin cut-off (obtained in **b**) and median values, a multinomial regression analysis adjusted for age and gender was conducted to calculate odds ratios (ORs) and 95 % confidence intervals (CI), represented by the *icons* and *bars*, respectively (**c**). A p-value <0.05 was considered statistically significant. *Mild mal* mild malaria group, *hyperbil* hyperbilirubinaemia group, *sev mal* severe malaria group

IFN-γ, TNF, CCL2, CCL4, GCSF, GMCSF) and inflammatory ratios (TNF/IL-10, IFN-γ/IL-10 and (TNF + IFN-γ)/IL-10) were used to build a heat map to identify unique signatures that could highlight differences between the study groups in a hierarchical cluster analysis (Fig. 2a). Among the clinical groups evaluated, individuals with severe malaria exhibited the highest median concentrations of the majority of the immune markers (GCSF, IFN-γ, IL-7, IL-2, IL-12p70, IL-13, IL-17, TNF, IL-4, IL-8, IL-6, IFN-γ/IL-10, TNF/IL-10, CCL2, and (TNF + IFN-γ)/IL-10; Fig. 2a). Subjects with mild malaria exhibited the highest plasma levels of IL-10 (Fig. 2a).

Multinominal regression analyses adjusted for age and gender revealed that severe malaria was associated with higher levels of IL-2 ($p = 0.025$) and IL-13 ($p = 0.018$) than malaria with hyperbilirubinaemia (Fig. 2b). Severe malaria was also associated with higher levels of IL-1β ($p = 0.008$), IL-2 ($p = 0.001$), IL-4 ($p = 0.041$), IL-12p70 ($p = 0.010$), IL-13 ($p = 0.004$), IFN-γ ($p = 0.041$), TNF ($p = 0.049$), IFN-γ/IL-10 ($p = 0.016$), TNF/IL-10 ($p = 0.016$) and (TNF + IFN-γ)/IL-10 ($p = 0.001$) than mild malaria (Fig. 2b). No differences in markers of immune expression were observed between the mild malaria and the hyperbilirubinaemia groups according to multinomial regression analyses (Fig. 2b).

A network analysis of immune response

Cytokine and chemokine levels were used to build networks representing possible interactions between the candidate biomarkers in each study group. The distributions of plasma concentrations of the cytokines and chemokines in each of the different clinical groups are provided (see Additional file 1). The network analysis revealed five negative correlations between candidate biomarkers in the study groups; four of these interactions were between IL-10 and its inflammatory ratios (IL-10 as a dividend). A majority of the statistically significant correlations observed were positive (Fig. 3a). However, the densities of the networks from each clinical group were different. The mild malaria group exhibited the highest density of interactions (network density: 0.605), which may be due to the higher number of individuals in this

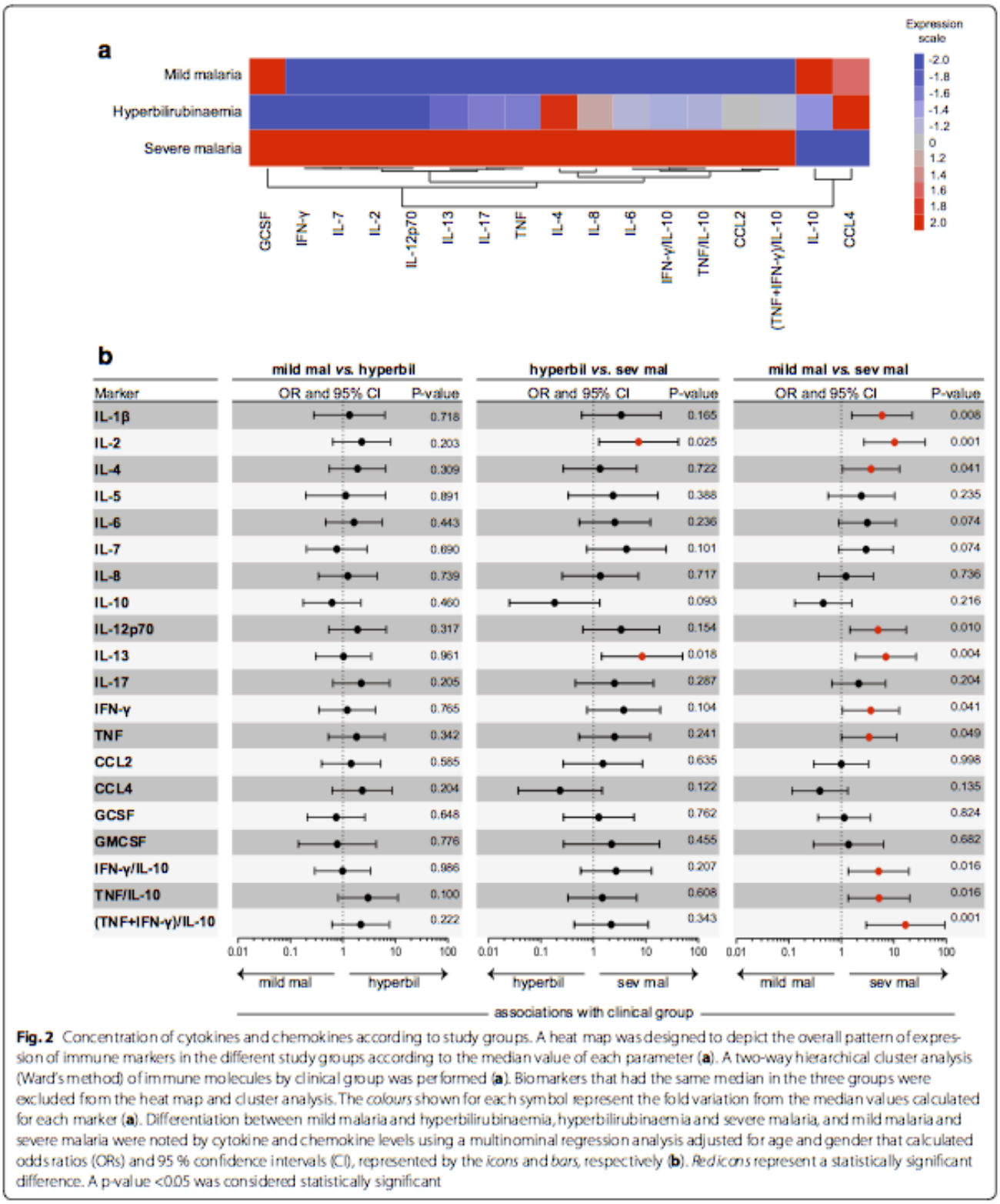


Fig. 2 Concentration of cytokines and chemokines according to study groups. A heat map was designed to depict the overall pattern of expression of immune markers in the different study groups according to the median value of each parameter (**a**). A two-way hierarchical cluster analysis (Ward's method) of immune molecules by clinical group was performed (**a**). Biomarkers that had the same median in the three groups were excluded from the heat map and cluster analysis. The *colours* shown for each symbol represent the fold variation from the median values calculated for each marker (**a**). Differentiation between mild malaria and hyperbilirubinaemia, hyperbilirubinaemia and severe malaria, and mild malaria and severe malaria were noted by cytokine and chemokine levels using a multinominal regression analysis adjusted for age and gender that calculated odds ratios (ORs) and 95 % confidence intervals (CI), represented by the *icons* and *bars*, respectively (**b**). *Red icons* represent a statistically significant difference. A p-value <0.05 was considered statistically significant

group compared to the other groups. The hyperbilirubinaemia group exhibited a network density of 0.284 and the severe malaria group exhibited the lowest density of 0.253. P-values and Spearman rank values for each correlation of immune biomarkers according to study groups are detailed (see Additional file 2).

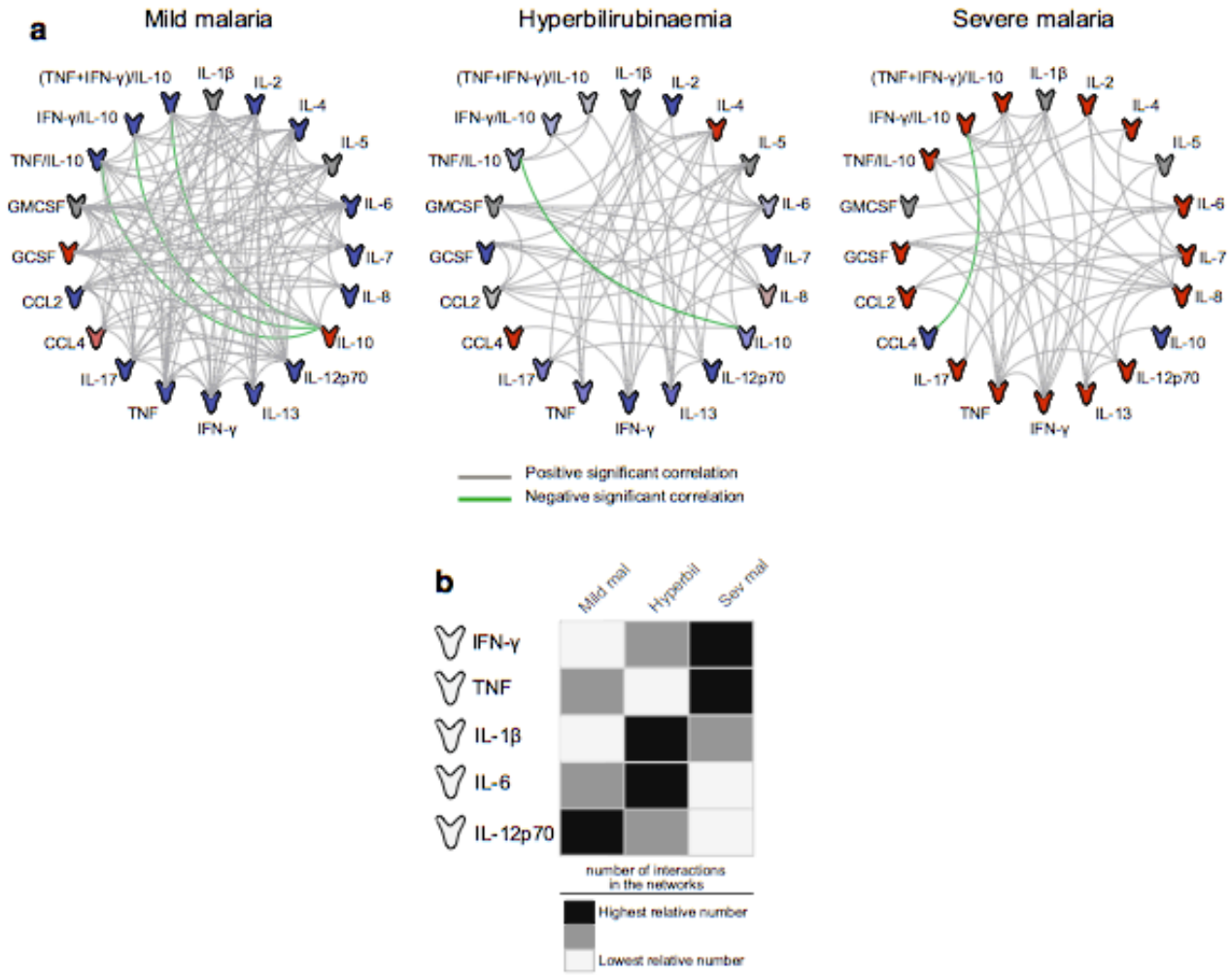


Fig. 3 Networks of immune-related biomarkers in mild malaria, hyperbilirubinaemia and severe malaria groups. Plasma levels of several immune-related (cytokines and chemokines) biomarkers were measured in subjects with mild malaria, hyperbilirubinaemia, and severe malaria. Each connecting line represents a significant interaction ($p < 0.05$) detected by Spearman's correlation test (**a**). *Grey lines* represent positive significant interactions and *green lines* represent negative significant correlations. *Icons* are colored according to their expression in the heat map shown in Fig. 2a. The five immune-related biomarkers with the highest number of interactions in all three groups were chosen (IFN-γ, TNF, IL-1β, IL-6, and IL-12p70) and the relative number of interactions of these biomarkers was calculated for each group (**b**). *Dark grey rectangles* represent the highest relative number of connections, *light dark rectangles* represent the medium relative number of connections, and *white rectangles* represent the lowest relative number of connections between molecules (**b**)

Of the 20 immune-related biomarkers and inflammatory ratios assessed in the network analyses, five cytokines (IFN-γ, TNF, IL-1β, IL-6, and IL-12p70) exhibited the most interactions (statistically significant Spearman correlations) across all groups. IFN-γ participated in 13.82 % of all interactions, TNF in 13.36 % of all interactions, IL-1β in 15.21 % of all interactions, IL-6 in 13.82 % of all interactions, and IL-12p70 in 12.44 % of all interactions (Fig. 3b). In order to assess if the number of network connections involving each of these cytokines could highlight differences among the clinical groups, the percentage of edges involving each molecule as a portion of the overall number of edges in the network was calculated. IFN-γ and TNF showed the highest relative number of network interactions in the severe malaria group and IL-1β and IL-6 showed the highest relative number of interactions in the hyperbilirubinaemia group (Fig. 3b). IL-12p70 showed the highest number of interactions in the mild malaria group (Fig. 3c). These results suggest that unique immune signatures involving plasma cytokine and chemokine levels highlight differences that differentiate mild malaria, malaria with hyperbilirubinaemia, and severe malaria.

Next, the interactions between clinical laboratory markers, parasitaemia, hepcidin levels, and immune-related molecules were evaluated (Fig. 4). As expected, in all study groups, HB levels were positively correlated with HT and ALT was positively correlated with AST (Fig. 4). Hepcidin was positively correlated with IL-6 ($r = 0.4339$; $p = 0.0015$), IL-10 ($r = 0.5154$; $p = 0.0002$), and parasitaemia ($r = 0.3032$; $p = 0.0096$) and negatively correlated with IFN-γ/IL-10 ($r = -0.4369$; $p = 0.0017$) and (TNF + IFN-γ)/IL-10 ($r = -0.3776$; $p = 0.0075$) in the mild malaria group. Hepcidin was positively correlated with parasitaemia ($r = 0.5912$; $p = 0.0260$) in the hyperbilirubinaemia group and with IFN-γ ($r = 0.5324$; $p = 0.0278$) in the severe malaria group. P-values and Spearman rank values for each correlation between immune biomarkers, laboratory measures, parasitaemia, and hepcidin levels are provided (see Additional file 3).

Discussion

Relationships between plasma markers of immune response and hepcidin levels during severe vivax malaria and malaria with hyperbilirubinaemia are largely unexplored. The results of the present study indicate that plasma hepcidin levels are higher in individuals with severe vivax malaria and individuals with hyperbilirubinaemia than in those with mild malaria; cut-off values of hepcidin can discriminate malaria outcomes. However, individuals with malaria with hyperbilirubinaemia had plasma cytokine and chemokine levels similar to those of individuals with mild malaria. Subjects with hyperbilirubinaemia exhibited a less robust inflammatory response than subjects with severe malaria, which reinforces the exclusion and adaptation of this condition in the most recent WHO malaria severity criteria [6]. Hepcidin was positively associated with IL-6, IL-10, and parasitaemia

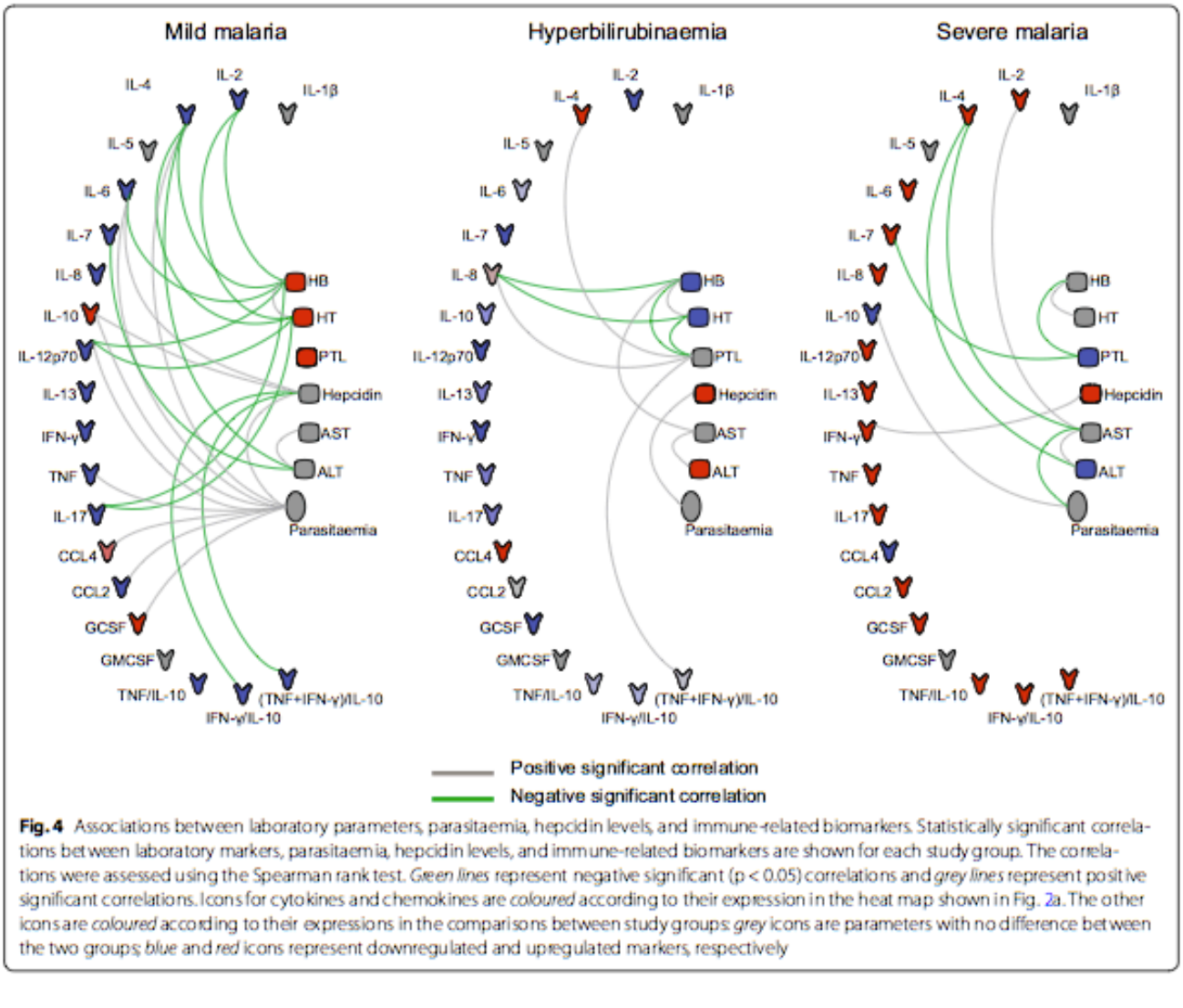


Fig. 4 Associations between laboratory parameters, parasitaemia, hepcidin levels, and immune-related biomarkers. Statistically significant correlations between laboratory markers, parasitaemia, hepcidin levels, and immune-related biomarkers are shown for each study group. The correlations were assessed using the Spearman rank test. *Green lines* represent negative significant ($p < 0.05$) correlations and *grey lines* represent positive significant correlations. Icons for cytokines and chemokines are *coloured* according to their expression in the heat map shown in Fig. 2a. The other icons are *coloured* according to their expressions in the comparisons between study groups: *grey* icons are parameters with no difference between the two groups; *blue* and *red* icons represent downregulated and upregulated markers, respectively

in the mild malaria group and with IFN-γ in the severe malaria group, which suggests a possible role of immunological regulation in the expression of this hormone.

Jaundice is a clinical outcome often associated with malaria infection, but the exact pathogenesis of this condition is not clearly understood [5]. The rupture of red blood cells, ischemia due to adherence of parasitized erythrocytes to the endothelial walls, and, to a lesser extent, hepatocellular dysfunction can contribute to hyperbilirubinaemia [9]. The present study confirms that individuals with hyperbilirubinaemia in the context of vivax malaria have low levels of PTL and high levels of ALT, which suggests that a degree of liver dysfunction associated with this condition might influence hepcidin expression by hepatocytes [12–16]. Hyperbilirubinaemia is not itself a complication of severe malaria, but it is associated with higher morbidity and mortality when it occurs concomitantly with at least one other complication [5, 8, 11]. In this study, individuals infected by *P. vivax* with hyperbilirubinaemia had lower levels of IL-2 and IL-13 than individuals with severe cases of malaria; no differences in immune response were observed between subjects with hyperbilirubinaemia and those with mild malaria. Even though the hyperbilirubinaemia group demonstrated an overall low immunoinflammatory status, the immune response may still have an important role in the pathogenesis of hyperbilirubinaemia. In the network analysis, IL-1β and IL-6 were highly associated with other inflammatory parameters. In fact, these markers may regulate the quality of the immune response and the high hepcidin levels associated with hyperbilirubinaemia. Levels of IL-6 have already been associated with severe malaria outcomes, and jaundice has been demonstrated to be independently linked to increased levels of this cytokine [18, 30]. Furthermore, IL -6 appears to be correlated with hepcidin levels during malaria infection [22, 25–27].

Traditionally, malaria by *P. vivax* has been considered a benign infection, but, in recent years, *P. vivax* malaria has been increasingly associated with severe disease [31–33]. The immune response, with its balance of anti- and pro-inflammatory mediators, seems to play an essential role in severe vivax malaria [2]. As expected, in the present study, individuals with severe vivax malaria exhibited higher concentrations of immune markers. This group also demonstrated a significant interaction between IFN-γ and TNF. Elevated levels of TNF and IFN-γ are related to severe forms of infection, such as cerebral malaria and severe anaemia in *P. falciparum* malaria [18, 33, 34]. In contrast, subjects with mild malaria displayed herein higher levels of IL-10 than subjects with severe malaria. These findings confirm previous studies that indicated a role of this regulatory cytokine in mild malaria outcomes [33, 35].

Hepcidin is the major regulator of iron levels, which it accomplishes by degrading the ferroportin receptor and inhibiting iron release from intracellular compartments. Mutations in the *ferroportin* gene (*SLC40A1*) have been linked to high iron stores and haemochromatosis in humans influencing hepcidin levels [29, 36, 37]. In this study population, the *SLC40A1* SNP (744 G > T) was rarely detected and it did not influence vivax malaria outcomes. Several studies have shown that hepcidin levels are increased in asymptomatic and symptomatic malaria caused by *P. falciparum* or *P. vivax* [21–24, 38]. In this study, higher plasma levels of hepcidin were observed in subjects with severe malaria and malaria with hyperbilirubinaemia than in subjects with mild malaria. The increased hepcidin levels in the malaria with hyperbilirubinaemia group may be a consequence of liver damage, hepatocellular dysfunction, and the immune response associated with hyperbilirubinaemia. Despite the possibility of liver dysfunction influencing hepcidin levels [18, 19] in malaria with hyperbilirubinaemia group, it was not observed a direct correlation between hepcidin and ALT or AST levels, and other liver biomarkers cannot be explored due to limitation of the volume of stored plasma.

Recently, hepcidin was discovered to be the best predictor of erythrocyte iron incorporation, which suggests a role for this hormone in the management of iron supplementation programmes [28]. Hepcidin cut-off values may also be used to distinguish severe malaria from mild malaria (without hyperbilirubinaemia) outcomes in endemic settings, as demonstrated in this study. Further, hepcidin cut-off values can be an additional tool (biomarker) in addition to the WHO criteria to distinguish severe or hyperbilirubinaemia patients from mild infection. Interestingly, some studies have reported that individuals with severe falciparum malaria, defined by severe anaemia and cerebral malaria, have low hepcidin levels [25–27]. Such differences in hepcidin concentrations may be explained by several factors. First, these studies measured hepcidin in children, whose degree of anaemia, which is defined by HB levels, is lower than adults in a majority of severe cases; second, cytokine levels in low-hepcidin severe malaria cases are lower than in uncomplicated cases, which suggests a lack of inflammation-driven stimulus for hepcidin production. In the present study, subjects with severe malaria had a robust pro-inflammatory response that may stimulate the expression of hepcidin.

Hepcidin is upregulated in response to several infectious and inflammatory conditions [39]. In a murine model, the production of hepcidin during a blood-stage infection can prevent a subsequent liver-stage infection; this inhibition was preserved in mice treated with

anti-IL-6 antibodies [20]. Recently, it was shown that IL-10 and IL-6 are increased in primary macrophages co-cultured with *P. falciparum*-infected erythrocytes; IL-10 seemed to induce hepcidin in macrophages, which was mediated by signal transducer and activator of transcription 3-phosphorylation [40]. Also, malaria-infected red blood cells induced hepcidin mRNA synthesis by peripheral blood mononuclear cells, which indicates the importance of circulatory immune cells on hepcidin production [19]. Hepcidin was positively correlated with IL-6 and IL-10 in the mild malaria group in the present study. IL-10 is a regulatory cytokine and the correlation between hepcidin and this cytokine can modulate the clinical disease observed in mild malaria cases. Hepcidin was also associated with parasitaemia in subjects with mild malaria and hyperbilirubinaemia, which agrees with the results of other studies [21, 23]. The association between parasite burden and inflammatory response may explain the relationship between hepcidin and parasitaemia, although a direct effect of the malaria parasite on hepatic and/or macrophage hepcidin production cannot be ruled out [23]. Hepcidin was positively correlated with IFN-γ in subjects with severe malaria in the present study, which further suggests a role of immune response in hepcidin regulation. IFN-γ is a proinflammatory cytokine involved in malaria pathogenesis and symptomatology [33], and the correlation between hepcidin and this cytokine may influence malaria severity. Interactions between immunological factors and hepcidin may be different according to malaria outcomes; however, the small sample sizes in the severe malaria and malaria with hyperbilirubinaemia groups may have underestimated these associations.

This study was limited because of a small number of subjects in the severe and hyperbilirubinaemia groups, which may have underestimated the associations between the markers studied herein. Further, unfortunately, others iron parameters (i.e. ferritin, transferrin receptor) were not analysed to better understand the relation of hepcidin, immune response and iron metabolism.

Conclusion

Hyperbilirubinaemia is a common clinical feature often associated with complications during malaria infection. The cytokine and chemokine responses in hyperbilirubinaemia are similar to the responses in uncomplicated malaria cases but less robust than the inflammatory responses observed in severe vivax malaria. Hepcidin levels are increased in cases of both severe malaria and malaria with hyperbilirrubinemia, and hepcidin levels are positively correlated with IL-6 and IL-10 in the mild malaria cases, and with IFN-γ in severe malaria subjects. These findings highlight the importance of immune response on hepcidin regulation. Hepcidin levels can be used to define iron supplementation programmes, but the findings from the present study suggest that hepcidin can also be used as a supplementary diagnostic marker of malaria infection.

Additional files

Additional file 1. Distribution of cytokines and chemokines in mild malaria, hyperbilirubinaemia and severe malaria groups.

Additional file 2. Correlation parameters between the immune biomarkers according to study groups.

Additional file 3. Correlation parameters between the immune biomarkers and laboratory measures, hepcidin and parasitaemia in the study groups.

Abbreviations

FMT-HVD: Fundação de Medicina Tropical Dr Heitor Vieira Dourado; PCR: polymerase chain reaction; RFLP: restriction fragment polymorphism; ELISA: enzyme-linked immunosorbent assay; HIV: human immunodeficiency virus; WHO: World Health Organization; IL-: Interleukin; IFN: interferon; TNF: tumour necrosis factor; CCL2: chemokine C–C motif ligand 2; CCL4: chemokine C–C motif ligand 4; GCSF: granulocyte colony stimulating factor; GMCSF: granulocyte macrophage colony stimulating factor; HB: haemoglobin; HT: haematocrit; PTL: platelets; AST: aspartate transaminase; ALT: alanine transaminase; OR: odds ratio; CI: confidence interval; HWE: Hardy-Weinberg equilibrium; AUC: area under the curve; ROC: receiver operating characteristic; SLC40A1: *ferroportin* gene.

Authors' contributions

VRRM performed the experiments, analysed the data and wrote the manuscript together with MSG and MBN. LCLS and GCG helped perform the experiments. BMLM was responsible for the field study and sampling. MVGL supervised the clinical study and sampling in Manaus. MBN conceptualized the study, and helped with data interpretation and the writing of the manuscript. All authors have read and approved the final version of the manuscript.

Author details

[1] Laboratório Integrado de Microbiologia e Imunoregulação (LIMI), Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Brazil. [2] Faculdade de Medicina, Universidade Federal da Bahia, Salvador, Brazil. [3] Fundação de Medicina Tropical Dr Heitor Vieira Dourado, Manaus, Brazil. [4] Universidade do Estado do Amazonas, Manaus, Brazil. [5] Instituto de Investigação em Imunologia, Instituto Nacional de Ciência e Tecnologia, São Paulo, Brazil.

Acknowledgements

The authors thank the team at FIOCRUZ-Bahia, Brazil, including Jorge Tolentino, Adorielze Leite, Nataly Alexandrino and Andrezza Kariny for help with logistics. The authors also thank Patricia Bozza and Edson Assis at FIOCRUZ-RJ for help with the multiplex assay experiment and Silvia Portugal for critical review. This work was supported by FIOCRUZ and FMT-HVD. MBN, MVGL and MSG are senior investigators from Conselho Nacional de Pesquisa e Tecnologia (CNPq), Brazil. VRRM, LCLS and BMLM are supported by CNPq. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 24 June 2015 Accepted: 5 October 2015
Published online: 14 October 2015

References

1. WHO. World malaria report 2014. Geneva: World Health Organization; 2014.
2. Mendonça VRR, Queiroz ATL, Lopes FM, Andrade BB, Barral-Netto M. Networking the host immune response in *Plasmodium vivax* malaria. Malar J. 2013;12:69.
3. Mendonça VR, Souza LC, Garcia GC, Magalhães BM, Lacerda MV, Andrade BB, et al. DDX39B (BAT1), TNF and IL6 gene polymorphisms and association with clinical outcomes of patients with *Plasmodium vivax* malaria. Malar J. 2014;13:278.
4. De Mendonça VRR, Goncalves MS, Barral-Netto M. The host genetic diversity in malaria infection. J Trop Med. 2012;2012:940616.
5. Anand AC, Puri P. Jaundice in malaria. J Gastroenterol Hepatol. 2005;20:1322–32.
6. WHO. Guidelines for the treatment of malaria. Second ed. Geneva: World Health Organization; 2010.
7. Lança EFC, Magalhães BML, Vitor-Silva S, Siqueira AM, Benzecry SG, Alexandre MAA, et al. Risk factors and characterization of Plasmodium vivax-associated admissions to pediatric intensive care units in the Brazilian Amazon. PLoS One. 2012;7:e35406.
8. Mishra SK, Patil SS, Satpathy SK, Mohanty S, Mohapatra DN. The influence of hyperbilirubinaemia on malaria-related mortality: an analysis of 1103 patients. Ann Trop Med Parasitol. 2004;98:555–8.
9. Saya RP, Debabrata G, Saya GK. Malarial hepatopathy and its outcome in India. N Am J Med Sci. 2012;4:449–52.
10. Treeprasertsuk S, Silachamroon U, Krudsood S, Huntrup A, Suwannakudt P, Vannaphan S, et al. Ursodeoxycholic acid and artesunate in the treatment of severe falciparum malaria patients with jaundice. J Gastroenterol Hepatol. 2010;25:362–8.
11. Tripathy R, Parida S, Das L, Mishra DP, Tripathy D, Das MC, et al. Clinical manifestations and predictors of severe malaria in Indian children. Pediatrics. 2007;120:e454–60.
12. Wilairatana P, Looareesuwan S, Charoenlarp P. Liver profile changes and complications in jaundiced patients with falciparum malaria. Trop Med Parasitol. 1994;45:298–302.
13. Abro AH, Ustadi AM, Abro HA, Abdou AS, Younis NJ, Akaila SI. Jaundice with hepatic dysfunction in *P. falciparum* malaria. J Coll Physicians Surg Pak. 2009;19:363–6.
14. Ahsan T, Ali H, Bkaht SF, Ahmad N, Farooq MU, Shaheer A, et al. Jaundice in falciparum malaria; changing trends in clinical presentation–a need for awareness. J Pak Med Assoc. 2008;58:616–21.
15. Fazil A, Vernekar PV, Geriani D, Pant S, Senthilkumaran S, Anwar N, et al. Clinical profile and complication of malaria hepatopathy. J Infect Public Health. 2013;6:383–8.
16. Shah S, Ali L, Sattar RA, Aziz T, Ansari T, Ara J. Malarial hepatopathy in falciparum malaria. J Coll Physicians Surg Pak. 2009;19:367–70.
17. Ganz T. Hepcidin and iron regulation, 10 years later. Blood. 2011;117:4425–33.
18. Day NP, Hien TT, Schollaardt T, Loc PP, Chuong LV, Chau TT, et al. The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. J Infect Dis. 1999;180:1288–97.
19. Armitage AE, Pinches R, Eddowes LA, Newbold CI, Drakesmith H. *Plasmodium falciparum* infected erythrocytes induce hepcidin (HAMP) mRNA synthesis by peripheral blood mononuclear cells. Br J Haematol. 2009;147:769–71.
20. Portugal S, Carret C, Recker M, Armitage AE, Gonçalves LA, Epiphanio S, et al. Host-mediated regulation of superinfection in malaria. Nat Med. 2011;17:732–7.
21. Howard CT, McKakpo US, Quakyi IA, Bosompem KM, Addison EA, Sun K, et al. Relationship of hepcidin with parasitemia and anemia among patients with uncomplicated *Plasmodium falciparum* malaria in Ghana. Am J Trop Med Hyg. 2007;77:623–6.
22. De Mast Q, van Dongen-Lases EC, Swinkels DW, Nieman A-E, Roestenberg M, Druilhe P, et al. Mild increases in serum hepcidin and interleukin-6 concentrations impair iron incorporation in haemoglobin during an experimental human malaria infection. Br J Haematol. 2009;145:657–64.
23. De Mast Q, Nadjm B, Reyburn H, Kemna EHJM, Amos B, Laarakkers CMM, et al. Assessment of urinary concentrations of hepcidin provides novel insight into disturbances in iron homeostasis during malarial infection. J Infect Dis. 2009;199:253–62.
24. De Mast Q, Syafruddin D, Keijmel S, Riekerink TO, Deky O, Asih PB, et al. Increased serum hepcidin and alterations in blood iron parameters associated with asymptomatic *P. falciparum* and *P. vivax* malaria. Haematologica. 2010;95:1068–74.
25. Casals-Pascual C, Huang H, Lakhal-Littleton S, Thezenas ML, Kai O, Newton CRJC, et al. Hepcidin demonstrates a biphasic association with anemia in acute *Plasmodium falciparum* malaria. Haematologica. 2012;97:1695–8.
26. Burté F, Brown BJ, Orimadegun AE, Ajetunmobi WA, Afolabi NK, Akinkunmi F, et al. Circulatory hepcidin is associated with the anti-inflammatory response but not with iron or anemic status in childhood malaria. Blood. 2013;121:3016–22.
27. Jonker FAM, Calis JCJ, Phiri K, Kraaijenhagen RJ, Brabin BJ, Faragher B, et al. Low hepcidin levels in severely anemic malawian children with high incidence of infectious diseases and bone marrow iron deficiency. PLoS One. 2013;8:e78964.
28. Prentice AM, Doherty CP, Abrams SA, Cox SE, Atkinson SH, Verhoef H, et al. Hepcidin is the major predictor of erythrocyte iron incorporation in anemic African children. Blood. 2012;119:1922–8.
29. Kasvosve I, Gomo ZAR, Nathoo KJ, Matibe P, Mudenge B, Loyevsky M, et al. Effect of ferroportin Q248H polymorphism on iron status in African children. Am J Clin Nutr. 2005;82:1102–6.
30. Kern P, Hemmer CJ, Van Damme J, Gruss HJ, Dietrich M. Elevated tumor necrosis factor alpha and interleukin-6 serum levels as markers for complicated *Plasmodium falciparum* malaria. Am J Med. 1989;87:139–43.
31. Kochar DK, Das A, Kochar SK, Saxena V, Sirohi P, Garg S, et al. Severe *Plasmodium vivax* malaria: a report on serial cases from Bikaner in northwestern India. Am J Trop Med Hyg. 2009;80:194–8.
32. Alexandre MA, Ferreira CO, Siqueira AM, Magalhães BL, Mourão MPG, Lacerda MV, et al. Severe *Plasmodium vivax* malaria, Brazilian Amazon. Emerg Infect Dis. 2010;16:1611–4.
33. Andrade BB, Reis-Filho A, Souza-Neto SM, Clarêncio J, Camargo LMA, Barral A, et al. Severe *Plasmodium vivax* malaria exhibits marked inflammatory imbalance. Malar J. 2010;9:13.
34. Lyke KE, Burges R, Cissoko Y, Sangare L, Dao M, Diarra I, et al. Serum levels of the proinflammatory cytokines interleukin-1 beta (IL-1beta), IL-6, IL-8, IL-10, tumor necrosis factor alpha, and IL-12(p70) in Malian children with severe *Plasmodium falciparum* malaria and matched uncomplicated malaria or healthy controls. Infect Immun. 2004;72:5630–7.
35. Liu Y, Chen Y, Li Z, Han Y, Sun Y, Wang Q, et al. Role of IL-10-producing regulatory B cells in control of cerebral malaria in *Plasmodium berghei* infected mice. Eur J Immunol. 2013;43:2907–18.
36. Montosi G, Donovan A, Totaro A, Garuti C, Pignatti E, Cassanelli S, et al. Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. J Clin Invest. 2001;108:619–23.
37. Wallace DF, Pedersen P, Dixon JL, Stephenson P, Searle JW, Powell LW, et al. Novel mutation in ferroportin1 is associated with autosomal dominant hemochromatosis. Blood. 2002;100:692–4.
38. Ayoya MA, Stoltzfus RJ, Spiekermann-Brouwer GM, Nemeth E, Traoré AK, Ganz T, et al. Hepcidin and *Plasmodium falciparum* malaria in anemic school children in Mali. Bull Soc Pathol Exot. 2009;102:219–20.
39. Spottiswoode N, Duffy PE, Drakesmith H. Iron, anemia and hepcidin in malaria. Front Pharmacol. 2014;5:125.
40. Huang H, Lamikanra AA, Alkaitis MS, Thézénas ML, Ramaprasad A, Moussa E, et al. Interleukin-10 regulates hepcidin in *Plasmodium falciparum* malaria. PLoS One. 2014;9:e88408.

5.6 MANUSCRITO VI

Unraveling the patterns of host immune responses in *Plasmodium vivax* malaria and dengue coinfection.

Este trabalho estuda a resposta imune (citocinas/quimiocinas séricas) e dados laboratoriais de indivíduos coinfectados com o virus da dengue e o *P. vivax*.

Resumo dos resultados: Um modelo de curva ROC (características de operação do receptor) multi-paramêtro, incluindo valores de hemoglobina, hematócrito, plaquetas e amino-transferases, teve uma alta performance para discriminar os diferentes grupos do estudo (malária, dengue e coinfeção malária/dengue). Um algoritmo diagnóstico usando as concentrações de plaquetas, hemoglobina e transaminases distinguiu com sucesso os participantes do estudo. Análise por rede da resposta imune demonstrou que o grupo coinfectado teve um perfil imune com papeis importantes para as citocinas TNF, IL-6 e IFN-γ.

Este trabalho foi publicado no periódico internacional *Malaria Journal.*

Mendonça et al. Malar J (2015) 14:315
DOI 10.1186/s12936-015-0835-8

MALARIA JOURNAL

RESEARCH Open Access

Unravelling the patterns of host immune responses in *Plasmodium vivax* malaria and dengue co-infection

Vitor R R Mendonça[1,2], Bruno B Andrade[1], Ligia C L Souza[1,2], Belisa M L Magalhães[3,4], Maria P G Mourão[3,4], Marcus V G Lacerda[3,4] and Manoel Barral-Netto[1,2,5*]

Abstract

Background: Concurrent malaria and dengue infection is frequently diagnosed in endemic countries, but its immunopathology remains largely unknown. In the present study, a large panel of cytokines/chemokines and clinical laboratory markers were measured in patients with *Plasmodium vivax* and dengue co-infection as well as in individuals with malaria or dengue mono-infections in order to identify biosignatures of each clinical condition.

Methods: Individuals from the Brazilian Amazon were recruited between 2009 and 2013 and classified in three groups: vivax malaria (n = 52), dengue (n = 30) and vivax malaria and dengue co-infection (n = 30). *P. vivax* malaria was diagnosed by thick blood smear and confirmed by PCR; dengue cases were detected by IgM ELISA or NS1 protein. The plasma levels of cytokines and chemokines were determined by multiplex assay.

Results: Individuals with malaria and dengue co-infection displayed lower levels of platelets and haemoglobin than those with malaria or dengue mono-infections ($p = 0.0047$ and $p = 0.0001$, respectively). The group of individuals co-infected exhibited the highest median concentrations of IFN-γ, IL-6, CCL4 than the mono-infected groups. Network analyses of plasma cytokines/chemokines revealed that malaria and dengue co-infection exhibits a distinct immune profile with critical roles for TNF, IL-6 and IFN-γ. Further, parasitaemia levels displayed positive significant interactions with IL-6, CCL4 and IL-10 in the group of patients co-infected with malaria and dengue. No differences were observed in distribution of dengue virus serotypes and *Plasmodium* parasitaemia levels between the groups.

Conclusions: The findings described here identify unique patterns of circulating immunological markers in cases of malaria and dengue co-infection and provide insights on the immunopathology of this co-morbid condition.

Keywords: Immune response, Co-infection, Dengue, *Plasmodium vivax*, Malaria

Background

Malaria and dengue fever are the most frequent arthropod-borne diseases in the world. Every year *Plasmodium* infection is responsible for around one million deaths, mainly in children [1]. It is estimated that two-fifths of the world population are at risk of dengue fever with 50–100 million cases each year worldwide [2, 3]. Both malaria and dengue fever exhibit dramatically similar geographic distribution (mostly in tropical and sub-tropical regions) and the detection of patients with concurrent malaria and dengue infections is not rare [4–19].

Previous studies have reported a frequent presence of malaria and dengue co-infections in different countries and implied that this fact creates challenges for reliable clinical diagnosis due to the overlap of major symptoms with malaria or dengue mono-infections [4, 6–8, 11, 12, 14, 16]. Recently, observations from a case series of patients with dual malaria and dengue infections performed at the Brazilian Amazon indicated that co-infection can potentially result in a more severe disease presentation [10]. The status of host immune activation

*Correspondence: mbarral@bahia.fiocruz.br
[5] Instituto de Investigação em Imunologia, Instituto Nacional de Ciência e Tecnologia, São Paulo, Brazil
Full list of author information is available at the end of the article

BioMed Central

© 2015 Mendonça et al. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

profile in patients with dengue and malaria co-infection, which may explain the clinical features of this condition, has not been systematically investigated.

The immunopathogeneses of dengue and malaria display common features, which include the production of multiple cytokines and the balance between pro-inflammatory and anti-inflammatory responses may regulate the clinical spectrum of these infections [20–25]. Importantly, circulating cytokines as well as other inflammatory mediators may be used as biomarkers for an early diagnosis or for prediction of unfavourable clinical deterioration and poor prognosis or treatment responses [26]. Moreover, understanding the key factors associated with increased morbidity may lead to development of host-directed therapy focused on the modulation of pathological immune responses and better clinical prognosis. The present study performs for the first time a detailed exploratory description of the systemic immune profile of individuals presenting with *Plasmodium vivax* malaria and dengue co-infection as well as in subjects with *P. vivax* or dengue mono-infections.

Methods

Study design and participants

Outpatients with an acute febrile syndrome who sought care in a reference hospital (Fundação de Medicina Tropical Doutor Heitor Vieira Dourado, FMT-HVD) in Manaus, in the Brazilian Amazon, were recruited between 2009 and 2013. Malaria individuals were diagnosed by blood thick smear and those with *P. vivax* confirmed by PCR were recruited. Dengue subjects were diagnosed by: NS1 and RT PCR (Kit Platelia™ Dengue NS1 Ag, Bio-Rad, France) in individuals with fewer than 6 days of fever, or by the detection of IgM ELISA as descrided by Kuno et al. [27] in individuals with more than 7 days of fever. All dengue-positive individuals were recruited and had the identification of virus serotype by RT PCR. Co-infected subjects with *P. vivax* malaria and dengue were also recruited. All patients with microscopic or molecular diagnosis of malaria caused by *Plasmodium falciparum* or *P. vivax* and *P. falciparum* co-infection (mixed infection), patients with serologic diagnosis of viral hepatitis (A, B, C, and D), HIV, and leptospirosis were excluded. Patients with complications of dengue or malaria according to WHO criteria [28] were excluded from this study. All malaria cases were treated following the guidelines of the National Foundation of Health, Brazil, with chloroquine for three days and primaquine (0.5 mg/kg/day) for 7 days. Dengue patients were treated according to their symptomatology. No individuals were treated for malaria or/and dengue 30 days before the blood collection and participation in this study.

Ethics statement

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all participants before enrolling into the study. This study was approved by the Ethics Committee of the FMT-HVD (protocol numbers: 2009/15243 and 39163/2012).

Plasma measurements

Blood was obtained by venopuncture at the study enrolment and heparinized plasma was separated by centrifugation and stored at −70 °C until use in immunoassays. Circulating levels of several cytokines and chemokines, including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17, IFN-γ, TNF, CCL2, CCL4, GCSF, GMCSF, were measured using a single multiplex assay according to the manufacturer's protocol (BIO-RAD, Hercules, CA, USA). The clinical laboratory markers haemoglobin (HB), haematocrit (HT), platelets (PTL), aspartate amino-transferase (AST) and alanine amino-transferase (ALT) were measured in fresh plasma/serum samples at the Clinical Laboratory facility from the FMT-HVD (Manaus, Brazil).

Network analysis

The inferential networks were generated from Spearman correlation matrices containing values of each biomarker measured in the plasma samples. The values were input in JMP 10.0 software (SAS, Cary, NC, USA). Each mediator is selected as a target, and the software performs a search within the other mediators for those that are correlated, with the target calculating a correlation matrix using Spearman rank tests. As a result, the features related to the selected target are linked. The links shown in the networks represent statistically significant Spearman rank correlations ($P < 0.05$). In order to analyse the structure of the biomarker networks, the network density was calculated, which is, in the context of this study, the ratio of the number of edges inferred in the network over the total number of possible edges between all pairs of nodes [24]. The density measure is defined as follows: $\text{density} = L/(N\ (N-1)/2)$, in which L is the number of observed edges (i.e., Spearman correlations with $P < 0.05$) and N is the total number of the nodes in the network. The density is normalized, ranging between 0 (no edges in the network) and 1 (all possible edges present). The networks figures were customized using the Ingenuity Systems Pathway Analysis software (Ingenuity Systems, Redwood City, CA, USA) and Adobe Illustrator (Adobe Systems Inc.).

Data analysis

In the exploratory analysis of the data, frequency tables were constructed and the Chi square test was applied to evaluate the association between categorical variables. The continuous variables were tested for Gaussian distribution within the total sample using D'Agostino and Pearson omnibus normality test. All variables were not under normal distribution, and non-parametric tests were used instead. In this context, Kruskal–Wallis with Dunn's multiple comparison (when three groups were compared) or Mann–Whitney tests (when two groups were compared) were used to assess the differences between the clinical groups. Multinomial regression analyses adjusted for age and gender were performed to test associations between the laboratory measures (below or above the median values of the entire study population) and the different clinical conditions evaluated (malaria, dengue or co-infection). A hierarchical cluster analysis using the Ward's method was performed to test if a combination of different immune-related biomarkers could cluster the study groups separately. The statistical analyses were performed using the programs GraphPad Prism 6.0 (GraphPad Software Inc., USA), SPSS 19.0 (IBM, Armonk, NY, USA) and JMP 11.0 (SAS, Cary, NC, USA). A *p* value lower than 0.05 was considered statistically significant.

Results

Characteristics of the study participants

After clinical and microbiological assessments, individuals were grouped as vivax malaria (n = 52), dengue (n = 30) and co-infected vivax malaria/dengue (n = 30). Most participants from the malaria group were male (80.77 %, n = 42) while the groups of co-infected and dengue patients exhibited a predominance of females (70.00 %, n = 9 for both; p < 0.0001). There were no differences with regard to age between the groups (p = 0.0724; Table 1) and also no statistically significant discrepancy in parasitaemia levels between the groups of individuals infected with *P. vivax* (p = 0.4912; Table 1). Moreover, the majority of individuals infected with dengue had DENV2 serotype with no differences in distribution of virus serotypes in the groups of individuals with dengue or dengue and malaria co-infection (Table 1).

Differential expression of clinical laboratory parameters reveals unique patterns of associations with infection status

Univariate analyses revealed that individuals co-infected with malaria and dengue exhibited lower levels of HB and PTL than those with malaria or dengue mono-infections (p = 0.0047 and p = 0.0001, respectively; Table 1). On the other hand, plasma AST levels were elevated whereas ALT concentrations were decreased in individuals with malaria mono-infection compared to those with dengue or co-infection (p = 0.0186 and p < 0.0001, respectively; Table 1). Multinominal regression analyses adjusted for age and gender uncovered that higher levels of HB (adjusted OR: 23.344 95 % CI 2.534–215.023, p = 0.005) and PTL (adjusted OR: 8.065 95 % CI: 1.527–42.612, p = 0.014) were associated with dengue when compared to malaria (Fig. 1). Furthermore, higher levels of HB (adjusted OR: 6.264 95 % CI 1.535–25.553, p = 0.011) and PTL (adjusted OR: 21.471 95 % CI 3.077–149.827, p = 0.002) were associated with dengue mono-infection compared with dengue and malaria co-infection (Fig. 1). Higher levels of AST (unadjusted OR: 3.030 95 % CI 1.160–7.914, p = 0.024) and low levels of ALT (adjusted OR: 0.219 95 % CI 0.069–0.695, p = 0.010) were associated with malaria mono-infection compared with the malaria/dengue co-infection condition (Fig. 1).

Networking the immune response

A panel of 17 cytokines and chemokines was used to build networks demonstrating the interactions between the candidate biomarkers in each group (Fig. 2a). The distributions of plasma concentrations of each cytokine or chemokine amongst the different clinical groups are provided (see Additional file 1). The network analysis revealed an absence of negative correlations between the candidate biomarkers in each one of the clinical groups and only statistically significant positive correlations were detected (Fig. 2a). Strikingly, the densities of the networks from each clinical group were dramatically different (Fig. 2a). The group of malaria mono-infection exhibited highest density of interactions (network density: 0.661) followed by the groups of co-infected patients (network density: 0.4338) and dengue mono-infection (network density: 0.147) (Fig. 2a). P values and Spearman rank values for each correlation between the immune biomarkers according to study groups are detailed (see Additional file 2). Moreover, the simultaneous assessment of several immune-related markers revealed relative differences in plasma concentrations that resulted in unique biosignatures, which could highlight differences between the study groups in an hierarchical cluster analysis (Fig. 2b). Amongst the clinical groups evaluated, the group of individuals with malaria and dengue co-infection exhibited the highest median concentrations of IFN-γ, IL-6, CCL4 (Fig. 2b). The group of malaria mono-infected patients exhibited a biosignature composed by higher levels of IL-10 and CCL2 whereas the group of dengue mono-infected individuals displayed a signature with high expression of IL-4, IL-7 and Il-12 in plasma (Fig. 2b). Furthermore, TNF was found elevated in both groups of malaria mono-infection and co-infection with

Table 1 Demographic characteristics and laboratory measures of the participants

	Malaria (n = 52)	Co-infection Mal/Deng (n = 30)	Dengue (n = 30)	P value		
				All groups	Malaria vs. co-infection	Dengue vs. co-infection
Male-no. (%)	42 (80.77)	09 (30.00)	09 (30.00)	<0.0001[a]	<0.0001[a]	1.000[a]
Median (IQR) age (year)	36.00 (26.25–43.75)	31.11 (20.80–44.74)	42.50 (30.00–52.25)	0.0724[b]	0.4471[b]	0.0574[b]
Median (IQR) of parasitaemia (parasites/uL)	3,022 (985.2–9,313)	4,262 (1,595–12,199)	–	–	0.4912[b]	–
Dengue serotypes-no. (%)						
DENV1		3 (10.00)	1 (3.33)	–	–	0.2247[a]
DENV2		18 (60.00)	21 (70.00)			
DENV3		1 (3.33)	4 (13.33)			
DENV4		8 (26.67)	4 (13.33)			
Median of laboratory measures (IQR)						
Haemoglobin (g/dL)	13.20 (12.50–14.20)	12.95 (11.90–14.45)	15.00 (13.40–15.95)	0.0047[b]	0.4819[b]	0.0038[b]
Haematocrit (%)	43.35 (40.43–45.98)	42.05 (37.80–45.98)	43.90 (40.65–46.90)	0.5803[b]	0.3732[b]	0.3817[b]
Platelets (by mm^3)	102,000 (65,000–131,500)	87,500 (59,000–114,250)	186,500 (124,000–229,750)	0.0001[b]	0.1079[b]	<0.0001[b]
AST (IU/L)	67.50 (50.00–91.00)	47.00 (31.50–72.50)	47.00 (28.00–147.00)	0.0186[b]	0.0048[b]	0.9315[b]
ALT (IU/L)	33.00 (20.00–49.75)	69.00 (46.00–95.50)	67.00 (29.50–119.00)	<0.0001[b]	< 0.0001[b]	0.9043[b]

IQR interquantile range.

[a] Categorized variables were compared using Chi square test or Fisher exact test.

[b] Continuous variables were compared using Mann–Whitney for two groups or Kruskal–Wallis test with Dunn's multiple comparison test for three groups or more.

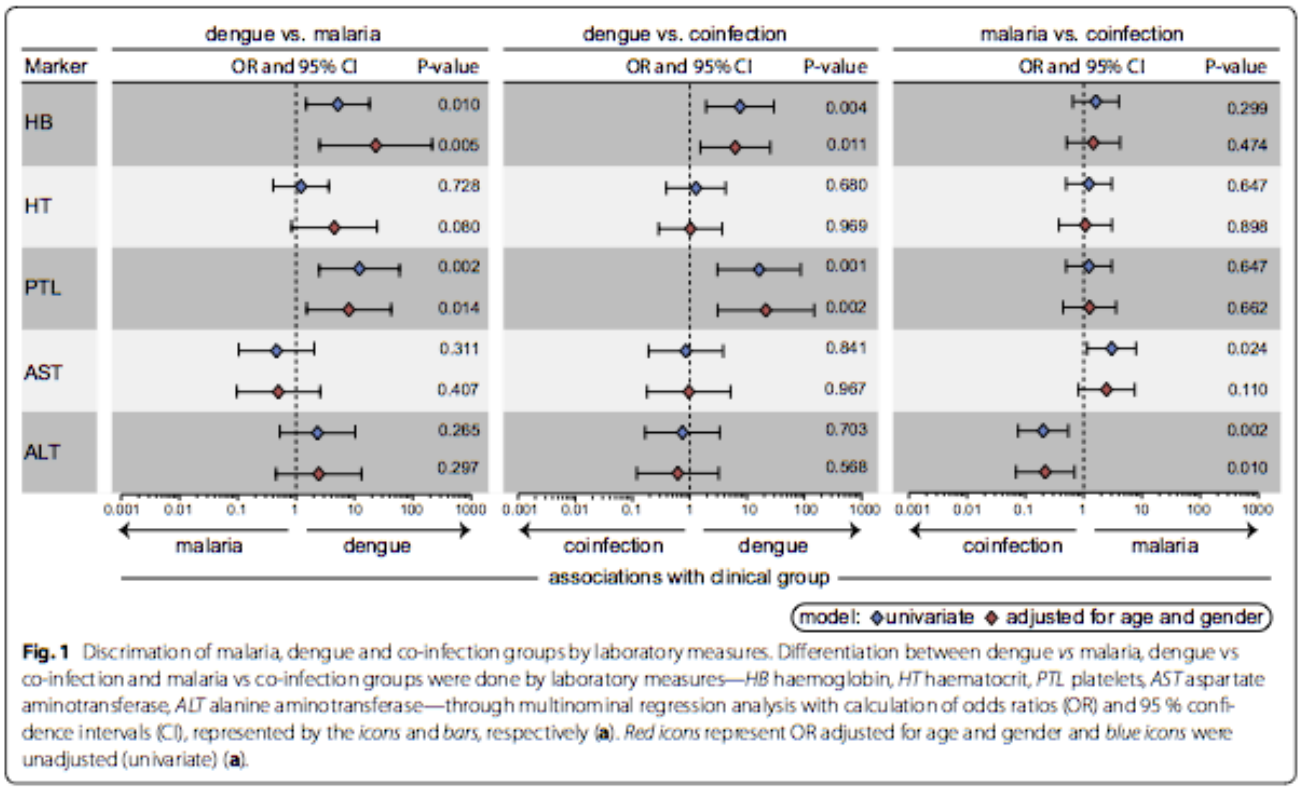


Fig. 1 Discrimation of malaria, dengue and co-infection groups by laboratory measures. Differentiation between dengue *vs* malaria, dengue vs co-infection and malaria vs co-infection groups were done by laboratory measures—*HB* haemoglobin, *HT* haematocrit, *PTL* platelets, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase—through multinominal regression analysis with calculation of odds ratios (OR) and 95 % confidence intervals (CI), represented by the *icons* and *bars*, respectively (**a**). *Red icons* represent OR adjusted for age and gender and *blue icons* were unadjusted (univariate) (**a**).

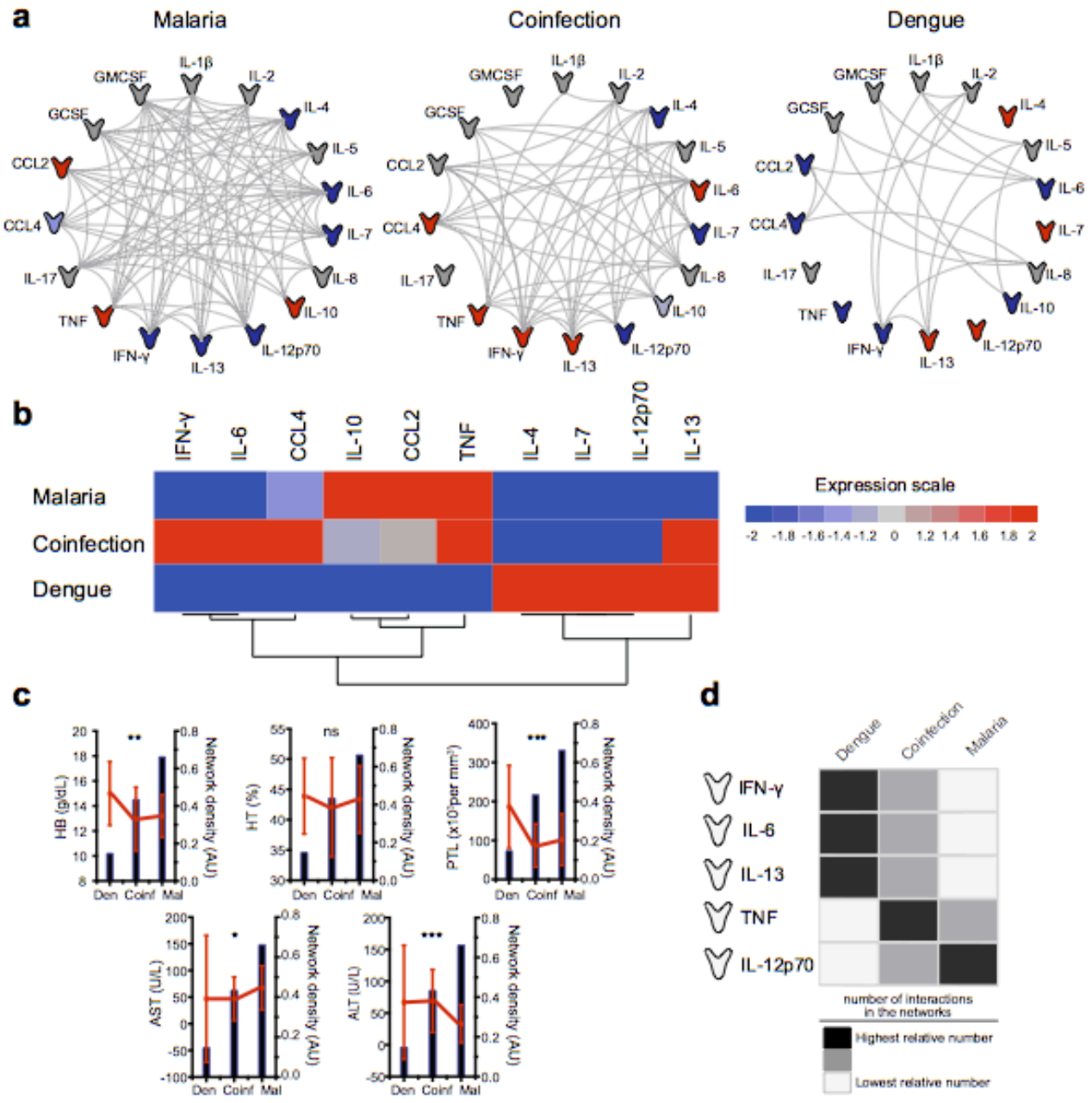


Fig. 2 Networks of candidate immune-related biomarkers during malaria, dengue or co-infection. Plasma levels of several immune-related (cytokines, chemokines) biomarkers were measured in malaria, dengue and co-infection subjects. Each connecting line represents a significant interaction ($P < 0.05$) detected by Spearman's correlation test (**a**). All interactions had positive correlations. A heat map was designed to depict the overall pattern of expression of immune markers in the different outcomes by the median value of each parameter (**b**). A two-way hierarchical cluster analysis (Ward's method) of immune molecules by clinical group was performed (**b**). Biomarkers that had the same median in the three groups were excluded from the heat map and cluster analysis. The *colours* shown for each *symbol* represent the fold variation from the median values calculated for each marker (**a**, **b**). The distribution of haemoglobin (HB), haematocrit (HT), platelets (PTL), aspartate aminotransferase (AST), alanine aminotransferase (ALT) in different clinical groups is shown in red symbols (medians and interquartile ranges) whereas the values for network densities are shown as *black bars* (**c**). The variation of HB, HT, PTL, AST, and ALT according to the groups was assessed using the Kruskal–Wallis test (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns = non-significant) (**c**). The five immune-related biomarkers with the highest number of interactions in all three groups were chosen (IFN-γ, IL-6, IL-13, TNF, and IL-12) and the relative number of interactions of these biomarkers was calculated according to each group (**d**). *Dark grey rectangles* represent the highest relative number of connections, *light grey rectangles* the medium relative number and *white rectangles* the lowest relative number of hits between molecules (**d**).

dengue whereas IL-13 was detected in higher amounts in the groups of dengue mono-infection and co-infection (Fig. 2b). While investigating the relationships between changes in clinical laboratory markers and the inflammatory environment assessed by network densities, it was observed that HB, PTL and ALT displayed a general trend to decrease in concentration values according to the increase of network's complexities (Fig. 2c). Nevertheless, AST levels tended to increase following the density of correlations between the markers in the groups (Fig. 2c). No significant difference was observed in variations of HT levels and its associations with network densities (Fig. 2c).

Amongst the 17 immune-related biomarkers assessed in the network analyses, five cytokines exhibited the highest number of interactions (statistically significant Spearman correlations) when all the study groups were considered together: IFN-γ (participated in 7.40 % of all interactions), IL-6 (8.88 % of all interactions), IL-13 (7.10 % of all interactions), TNF (6.50 % of all interactions) and IL-12p70 (6.50 % of all interactions) (Fig. 2d). In order to assess if the number of network connections involving each one of these five cytokines could highlight differences between the clinical groups, the percentage of edges involving with each molecule related to the overall number of edges in the network was calculated. Interestingly, IFN-γ, IL-6 and IL-13 displayed the highest relative number of network interactions in the group of dengue mono-infected patients, whereas IL-12p70 exhibited the highest relative number of interactions in the malaria mono-infection group (Fig. 2d). TNF exhibited the highest number of interactions in the group of patients with malaria and dengue co-infection (Fig. 2d). These results argue that unique immune signatures involving plasma cytokine levels are able to highlight differences that distinguish malaria, dengue or dual malaria and dengue infection.

The next step was to uncover the interactions between clinical laboratory markers and the immune-related molecules. In all the study groups, HB exhibited positive associations with HT whereas ALT exhibited positive correlations with AST (Fig. 3a). It was found that HB and HT displayed several negative significant interactions mainly with IL-4, IL-5, IL-12p70, and IL-17, whereas ALT interacted negatively with IL-4 and IL-7 in the malaria group (Fig. 3a). In the group of patients with malaria and dengue co-infection, HB and HT displayed negative associations with IL-7, whereas AST exhibited positive interactions with CCL2, IL-13 and IL-8 (Fig. 3a). Noteworthy, it was observed that only in the dengue mono-infection group did PTL display interactions with immune markers (positive interactions with GMCSF, GCSF and IL-8), suggesting a major role for this molecule in this group (Fig. 3a). Furthermore, AST and ALT displayed negative associations with TNF in the network of the dengue mono-infection group (Fig. 3a). In the *P. vivax*-infected groups, the associations between parasitaemia and the immune markers were also studied (Fig. 3b). It was observed that *P. vivax* parasitaemia displayed positive significant interactions with IL-6, CCL4 and IL-10 in the group of patients co-infected with malaria and dengue, while this parameter exhibited several positive correlations with many immune markers (GCSF, CCL2, CCL4, TNF, IL-12p70, IL-10, IL-6, and IL-4) in the group of malaria mono-infected subjects, suggesting a major role for parasitaemia in the immune profile in this clinical condition (Fig. 3b). P values and Spearman rank values for each correlation between the immune biomarkers and laboratory measures or parasitaemia are provided (see Additional file 3).

Discussion

Malaria or dengue immunology has been studied extensively in a diverse range of scenarios, however, the profile of the immune responses in individuals co-infected with *Plasmodium* and dengue virus has not been systematically explored. In the present study, a large panel of cytokines and chemokines as well as several clinical laboratory markers used to assess degree of disease severity and inflammation-driven tissue damage have been investigated in plasma samples of patients with malaria, dengue or co-infection in order to identify immune signatures associated with each infection status. Network analyses revealed that cases of malaria and dengue co-infection exhibit a unique immune profile with a special role for TNF, IL-6, IFN-γ, and IL-7. In addition, the analysis herein further revealed a signature profile in which *P. vivax* parasitaemia levels display positive significant interactions with IL-6, CCL4 and IL-10 in patients with this co-infection, which was not observed in malaria mono-infected individuals.

Some studies have studied laboratory measurements in subjects with dual malaria and dengue infections [4, 6, 11]. HB and PTL are usually shown to be decreased in individuals co-infected with malaria and dengue and AST has been shown to be higher in individuals with malaria compared with co-infected cases [4, 6, 11]. These findings are similar to the results described in the present study. When dengue was compared to malaria mono-infection or co-infected cases, the former had higher values of HB and PTL. Malaria infection is frequently associated with anaemia as its causative parasite has a blood stage and causes intense intravascular haemolysis. One major complication associated with both dengue and malaria disease is thrombocytopaenia and the findings from the present study indicate that *P. vivax* infection may cause

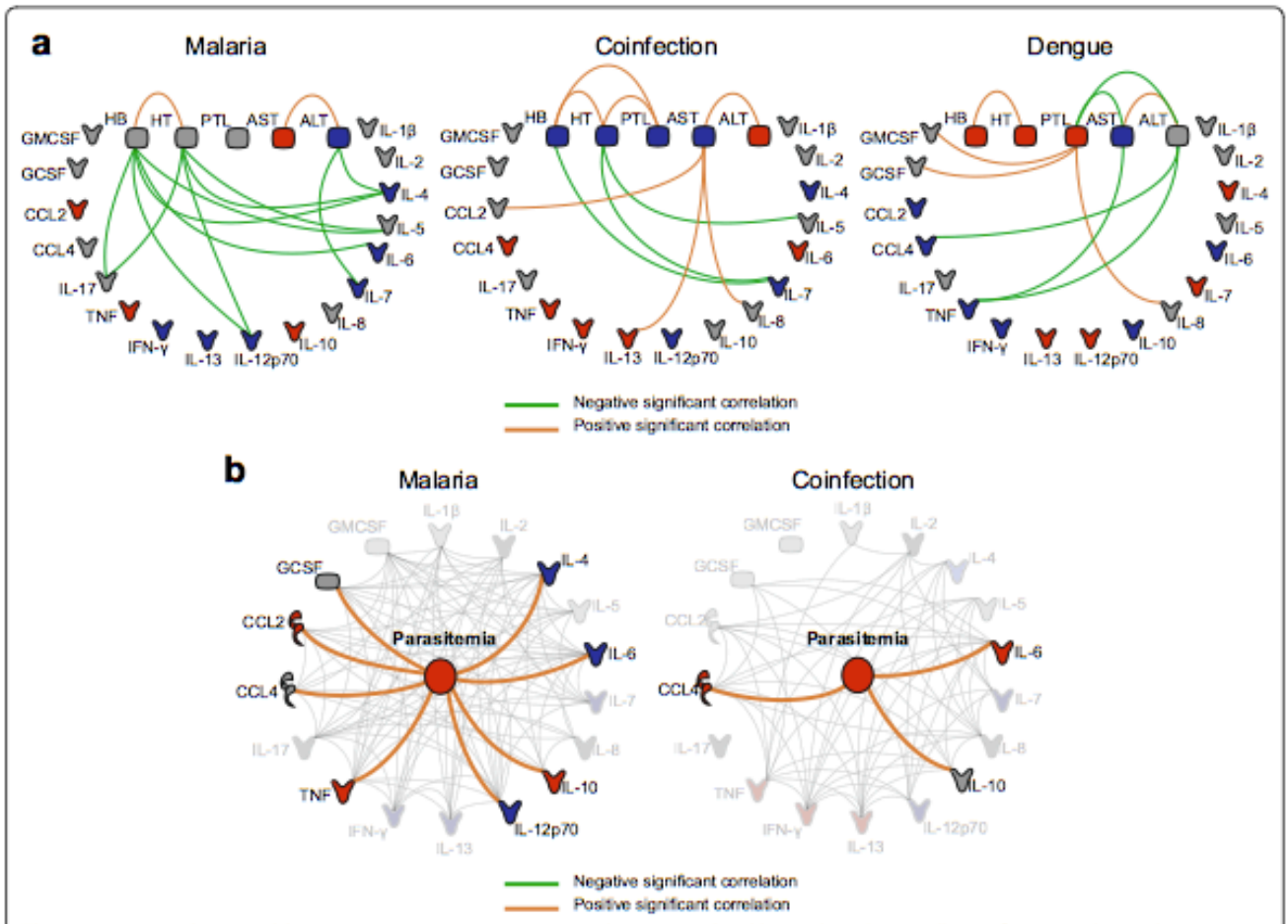


Fig. 3 Associations between laboratory parameters, parasitaemia and immune-related biomarkers. Statistically significant correlations between laboratory markers (**a**) or parasitaemia (**b**) and immune-related biomarkers are shown for the different groups. The correlations were assessed using the Spearman rank test. The interactions involving parasitaemia shown in (**c**) are plotted on top of the host interactome. *Green lines* represent negative significant ($P < 0.05$) correlations and *orange lines*, positive significant correlations.

more severe thrombocytopaenia (with lowest values in co-infected cases) compared to dengue fever mono-infection [29–31]. Other studies have also reported that platelet counts were lower in malaria patients than dengue patients [15, 32]. On the other hand, laboratory markers assessed in the current study showed similar values between malaria mono-infection and cases of malaria and dengue co-infection, with a major difference in ALT levels (and in less extension to AST), which were more elevated in the patients with co-infection, and no significant difference was observed in parasitaemia levels. The relationship of AST and ALT and parasitaemia with clinical manifestations of mono- or co-infection of malaria and dengue is not well established. A previous study showed higher levels of AST and ALT and parasitaemia in malaria mono-infection compared to malaria and dengue co-infection [11]. Nevertheless, another report showed no differences in concentrations of AST and ALT as well as levels of parasitaemia between malaria mono-infected patients and those with malaria and dengue co-infection [6]. Differences in patient populations and parasite strains could explain these discrepancies. Parasitaemia levels may have an important role in predicting hepatic damage and need to be considered in malaria-infected subjects [25].

Heightened levels of TNF and IFN-γ have been systematically associated with increased clinical disease severity in malaria or dengue fever in many case series [20, 21, 24, 25, 33–35]. An important role for TNF and IFN-γ in the onset of malaria symptomatology as well as in pathological processes associated with platelet consumption, endothelial cell activation and haemorrhagic manifestations during dengue fever have been described previously [33, 36]. In the present study, increased TNF levels observed in the group of patients with malaria and dengue co-infection compared to malaria or dengue mono-infections, together with the significantly higher number of interactions in the cytokine/chemokine

networks, argue that this cytokine may play a critical role in the pathogenesis of malaria and dengue fever comorbid condition. Noteworthy, *TNF* polymorphisms are common and may play a role in TNF levels in the context of malaria [37]. Moreover, amongst the clinical groups assessed herein, cases of malaria and dengue co-infection also exhibited the highest values of IFN-γ and IL-6. IL-6 has been implicated in the pathogenesis of severe cases of dengue as this cytokine enhances the production of anti-platelet or anti-endothelial cell auto-antibodies, as well as the induction of tissue plasminogen activator, leading to increased risk for bleeding [38, 39]. These findings on immune markers support the idea that co-infected cases may present with a more severe inflammation milieu and disease status compared to malaria or dengue mono-infections. Recently, it has been described that individuals with concurrent dengue fever and malaria from the Brazilian Amazon and French Guiana exhibited more severe disease clinical presentation than mono-infections [6, 9, 10].

In the present study, plasma levels of IL-4, IL-7, IL-12p70, and IL-13 were more elevated in subjects purely with dengue fever than in those with malaria mono-infection or malaria and dengue co-infection. Noteworthy, IL-12p70 has been previously associated with severity and protection during malaria [40–43], also polymorphism in this gene can influence this cytokine production during malaria [44]. IL-12p70 had the highest number of interacions in the malaria group and seems to influence the inflammatory milieu in this group. In addition, IL-13 had the highest relative number of interactions in the cytokine/chemokine network of the group of dengue mono-infection. IL-4 and IL-13 plasma levels were also previously found to be higher in dengue fever compared to malaria cases [23]. The increase in plasma expression of Th2 cytokines (i.e., IL-4, IL-13) may be associated with augmentation of vascular permeability and vascular leakage as seen in dengue haemorrhagic fever [45, 46]. Regarding malaria mono-infection, IL-10 and CCL2 were the immune markers with more relevant elevations in plasma. Maneekan et al. have described that IL-10 was statistically higher in malaria patients than in those with dengue fever [23]. Furthermore, IFN-γ/IL-10 ratio has been reported to increase proportionally to malaria clinical severity [25], and it is possible that cases of malaria and dengue co-infection presenting with lower levels of IL-10 and higher levels of IFN-γ may be reflected in an increased disease severity. Further studies, including a broader clinical spectrum of the infections explored here, are necessary to address this question. In a study about the immune profile of dengue fever and parvovirosis, high levels of CCL2 tended to be associated with parvovirus B19 infection as the same way as observed herein with malaria infection [47]. Therefore, different infectious agents disease may stimulate specific features of the host immune responses, which may result in unique immune signatures.

Although the group of individuals co-infected with malaria and dengue displayed an intermediate density of the immune network compared to the other clinical groups, this group displayed the lowest values of HB and PTL, thus indicating that the relationships between the inflammatory markers may not be influenced directly by the levels of these clinical laboratory markers. However, a potential role for HB and HT in regulation of the inflammatory environment in malaria and dengue comorbidity could not be entirely discarded, and the network findings indicate that these parameters were negatively correlated with IL-7 in this group. It has been reported that IL-7 plays an important role in CD4+ T cell immune responses against the dengue virus and this cytokine is also useful for maintaining the growth and antigen-specific cytotoxic activity of CD4+ human cytotoxic T lymphocyte clones [48]. In addition, AST seemed to be highly influenced by immune molecules in the malaria and dengue co-infected patients as this enzyme was positively correlated with CCL2, IL-13 and IL-8.

In the context of laboratory and immune markers correlations, the group of individuals presenting with malaria mono-infection displayed significant negative correlations between ALT and IL-4 and IL-7. Liver enzymes are highly inducible by the activation of immune responses and ALT was also correlated with TNF and superoxide dismutase-1 (SOD-1) levels in another case series of vivax malaria [49]. Furthermore, HB and HT had several negative interactions with inflammatory mediators (IL-17, IL-12, IL-5, IL-4) in the malaria group. These interactions may be explained as HB and HT levels as well as the immune response are directly influenced by intravascular haemolysis of red blood cell rupture upon *Plasmodium sp.* replication. Noteworthy, PTL positively interacted with immune markers (GCSF, GMCSF and IL-8) only in the group of patients with dengue mono-infection. It has been reported that exposure of monocytes from healthy volunteers to platelets from patients with dengue induced the secretion of IL-8 [50]. Thrombocytopaenia is often described in dengue fever (about of 23.6 % of cases) and it may play a role along with immune mediators in dengue immunopathology [51].

Parasitaemia was not different between the groups of individuals with malaria mono-infection and malaria and dengue co-infection, similarly to previous studies [6]. However, the network analysis uncovered that parasitaemia displayed more statistically significant interactions with cytokines/chemokines in the malaria mono-infection than in the group of co-infected patients.

The immune response of individuals with concurrent dengue and malaria infections is also influenced by dengue viruses and this may explain the lower number of interactions of parasitaemia in this group. Moreover, one limitation of the present report was that some co-infected individuals could have had undiagnosed asymptomatic malaria, which can sometimes be diagnosed only with molecular methods. It is well described that asymptomatic malaria individuals control parasitaemia to very low levels (sometimes only detectable by molecular techniques) and this clinical immunity results in a reduced intensity of the inflammatory response when compared to symptomatic malaria cases [24, 25].

Conclusion

Altogether, the findings of the present study indicate that concurrent malaria and dengue infections may cause a more severe disease compared to mono-infections as observed by laboratory and immune markers profiles. Overall, malaria and dengue co-infection displayed lower levels of platelets and haemoglobin and a specific immune signature with a special role for TNF compared to dengue or malaria. This detailed description of the immune response profile in subjects with malaria and dengue fever co-infection and the results depicted herein shed light into the immunopathology of this comorbid condition. Further prospective studies with larger samples and experimental models would be necessary to investigate the key mechanisms resulting in the biosignatures identified here in patients with malaria and dengue co-infection.

Additional files

Additional file 1. Distribution of cytokines and chemokines according to malaria, co-infection and dengue groups.

Additional file 2. Correlation parameters between the immune biomarkers according to study groups.

Additional file 3. Correlation parameters between the immune biomarkers and laboratory measures or parasitaemia in the study groups.

Abbreviations

FMT-HVD: Fundação de Medicina Tropical Dr Heitor Vieira Dourado; PCR: polymerase chain reaction; NS1: non-structural protein 1; RT PCR: real-time polymerase chain reaction; IgM: immunoglobulin M; ELISA: enzyme-linked immunosorbent assay; HIV: human immunodeficiency virus; WHO: World Health Organization; IL-: interleukin; IFN: interferon; TNF: tumour necrosis factor; CCL 2: chemokine C–C motif ligand 2; CCL4: chemokine C–C motif ligand 4; GCSF: granulocyte colony stimulating factor; GMCSF: granulocyte macrophage colony stimulating factor; HB: haemoglobin; HT: haematocrit; PTL: platelets; AST: aspartate transaminase; ALT: alanine transaminase; DENV: dengue virus; OR: odds ratio; CI: confidence interval; CD: cluster of differentiation; SOD-1: superoxide dismutase 1.

Authors' contributions

VRRM performed the experiments, analysed the data, and wrote the manuscript together with BBA and MBN. LCLS helped perform the experiments. BMLM was responsible for the field study and sampling. MVGL and MPGM supervised the clinical study and sampling in Manaus. MBN conceptualized the study, and helped with data interpretation and the writing of the manuscript. All authors read and approved the final manuscript.

Author details

[1] Laboratório Integrado de Microbiologia e Imunoregulação (LIMI), Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Brazil. [2] Faculdade de Medicina, Universidade Federal da Bahia, Salvador, Brazil. [3] Fundação de Medicina Tropical Dr. Heitor Vieira Dourado, Manaus, Brazil. [4] Universidade do Estado do Amazonas, Manaus, Brazil. [5] Instituto de Investigação em Imunologia, Instituto Nacional de Ciência e Tecnologia, São Paulo, Brazil.

Acknowledgements

The authors thank the research team at FIOCRUZ-Bahia, Brazil, Jorge Tolentino, Adorielze Leite, Nataly Alexandrino and Andrezza Karíny for help in logistics; and to Patricia Bozza and Edson Assis at FIOCRUZ-RJ for help in the multiplex assay experiment.

This work was supported by FIOCRUZ and FMT-HVD. MBN, MVGL and MPGM are senior investigators from Conselho Nacional de Pesquisa e Tecnologia (CNPq), Brazil. VRRM, LCLS and BMLM are supported by CNPq. Part of the work of BBA is supported by the Fundação José Silveira, Salvador, Bahia, Brazil. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

Received: 24 June 2015 Accepted: 4 August 2015
Published online: 14 August 2015

References

1. WHO (2014) World Malaria Report 2014. World Health Organization, Geneva
2. Gubler DJ (2002) The global emergence/resurgence of arboviral diseases as public health problems. Arch Med Res 33:330–342
3. Gubler DJ (2002) Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol 10:100–103
4. Abbasi A, Butt N, Sheikh QH, Bhutto AR, Munir SM, Ahmed SM (2009) Clinical features, diagnostic techniques and management of dual dengue and malaria infection. J Coll Physicians Surg Pak 19:25–29
5. Baba M, Logue CH, Oderinde B, Abdulmaleek H, Williams J, Lewis J et al (2013) Evidence of arbovirus co-infection in suspected febrile malaria and typhoid patients in Nigeria. J Infect Dev Ctries 7:51–59
6. Epelboin L, Hanf M, Dussart P, Ouar-Epelboin S, Djossou F, Nacher M et al (2012) Is dengue and malaria co-infection more severe than single infections? A retrospective matched-pair study in French Guiana. Malar J 11:142
7. Faruque LI, Zaman RU, Alamgir ASM, Gurley ES, Haque R, Rahman M et al (2012) Hospital-based prevalence of malaria and dengue in febrile patients in Bangladesh. Am J Trop Med Hyg 86:58–64
8. Hati AK, Bhattacharjee I, Mukherjee H, Bandyopadhayay B, Bandyopadhyay D, De R et al (2012) Concurrent dengue and malaria in an area in Kolkata. Asian Pac J Trop Med 5:315–317
9. Magalhaes BML, Alexandre MAA, Siqueira AM, Melo GC, Gimaque JBL, Bastos MS et al (2012) Clinical profile of concurrent dengue fever and *Plasmodium vivax* malaria in the Brazilian Amazon: case series of 11 hospitalized patients. Am J Trop Med Hyg 87:1119–1124
10. Magalhães BML, Siqueira AM, Alexandre MAA, Souza MS, Gimaque JB, Bastos MS et al (2014) *P. vivax* malaria and dengue fever co-infection: a cross-sectional study in the Brazilian Amazon. PLoS Negl Trop Dis 8:e3239
11. Mohapatra MK, Patra P, Agrawala R (2012) Manifestation and outcome of concurrent malaria and dengue infection. J Vector Borne Dis 49:262–265

12. Bin Mushtaq M, Qadri MI, Rashid A (2013) Concurrent infection with dengue and malaria: an unusual presentation. Case Rep Med 2013:520181
13. Park S-H, Lee M-J, Baek J-H, Lee W-C (2011) Epidemiological aspects of exotic malaria and dengue fever in travelers in Korea. J Clin Med Res 3:139–142
14. Santana VDS, Lavezzo LC, Mondini A, Terzian ACB, de Bronzoni RVM, Rossit ARB et al (2010) Concurrent dengue and malaria in the Amazon region. Rev Soc Bras Med Trop 43:508–511
15. Shah I, Katira B (2007) Clinical and laboratory profile of dengue, leptospirosis and malaria in children: a study from Mumbai. Arch Dis Child 92:561
16. Talarmin A, Peneau C, Dussart P, Pfaff F, Courcier M, de Rocca-Serra B, Sarthou JL (2000) Surveillance of dengue fever in French Guiana by monitoring the results of negative malaria diagnoses. Epidemiol Infect 125:189–193
17. da Silva-Nunes M, dos Malafronte RS, de Luz BA, de Souza EA, Martins LC, Rodrigues SG et al (2006) The acre project: the epidemiology of malaria and arthropod-borne virus infections in a rural Amazonian population. Cad Saude Publica 22:1325–1334
18. Thaha M, Pranawa, Yogiantoro M, Tanimoto M, Tomino Y (2008) Acute renal failure in a patient with severe malaria and dengue shock syndrome. Clin Nephrol 70:427–430
19. Yong KP, Tan BH, Low CY (2012) Severe falciparum malaria with dengue coinfection complicated by rhabdomyolysis and acute kidney injury: an unusual case with myoglobinemia, myoglobinuria but normal serum creatine kinase. BMC Infect Dis 12:364
20. Arias J, Valero N, Mosquera J, Montiel M, Reyes E, Larreal Y et al (2014) Increased expression of cytokines, soluble cytokine receptors, soluble apoptosis ligand and apoptosis in dengue. Virology 452–453:42–51
21. Bozza FA, Cruz OG, Zagne SMO, Azeredo EL, Nogueira RMR, Assis EF et al (2008) Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. BMC Infect Dis 8:86
22. Kumar Y, Liang C, Bo Z, Rajapakse JC, Ooi EE, Tannenbaum SR (2012) Serum proteome and cytokine analysis in a longitudinal cohort of adults with primary dengue infection reveals predictive markers of DHF. PLoS Negl Trop Dis 6:e1887
23. Maneekan P, Leaungwutiwong P, Misse D, Luplertlop N (2013) T helper (Th) 1 and Th2 cytokine expression profile in dengue and malaria infection using magnetic bead-based bio-plex assay. Southeast Asian J Trop Med Public Health 44:31–36
24. Mendonça VRR, Queiroz ATL, Lopes FM, Andrade BB, Barral-Netto M (2013) Networking the host immune response in *Plasmodium vivax* malaria. Malar J 12:69
25. Andrade BB, Reis-Filho A, Souza-Neto SM, Clarêncio J, Camargo LMA, Barral A et al (2010) Severe *Plasmodium vivax* malaria exhibits marked inflammatory imbalance. Malar J 9:13
26. Andrade BB, Barral-Netto M (2011) Biomarkers for susceptibility to infection and disease severity in human malaria. Mem Inst Oswaldo Cruz 106(Suppl):70–78
27. Kuno G, Gómez I, Gubler DJ (1987) Detecting artificial anti-dengue IgM immune complexes using an enzyme-linked immunosorbent assay. Am J Trop Med Hyg 36:153–159
28. WHO (2010) Guidelines for the treatment of malaria, 2nd edn. World Health Organization, Geneva
29. Mourão MPG, Lacerda MVG, Macedo VO, Santos JB (2007) Thrombocytopenia in patients with dengue virus infection in the Brazilian Amazon. Platelets 18:605–612
30. Coelho HCC, Lopes SCP, Pimentel JPD, Nogueira PA, Costa FTM, Siqueira AM et al (2013) Thrombocytopenia in *Plasmodium vivax* malaria is related to platelets phagocytosis. PLoS One 8:e63410
31. Lacerda MVG, Mourão MPG, Coelho HCC, Santos JB (2011) Thrombocytopenia in malaria: who cares? Mem Inst Oswaldo Cruz 106(Suppl):52–63
32. Kutsuna S, Hayakawa K, Kato Y, Fujiya Y, Mawatari M, Takeshita N et al (2014) The usefulness of serum C-reactive protein and total bilirubin levels for distinguishing between dengue fever and malaria in returned travelers. Am J Trop Med Hyg 90:444–448
33. Espina LM, Valero NJ, Hernández JM, Mosquera JA (2003) Increased apoptosis and expression of tumor necrosis factor-alpha caused by infection of cultured human monocytes with dengue virus. Am J Trop Med Hyg 68:48–53
34. Levy A, Valero N, Espina LM, Añez G, Arias J, Mosquera J (2010) Increment of interleukin 6, tumour necrosis factor alpha, nitric oxide, C-reactive protein and apoptosis in dengue. Trans R Soc Trop Med Hyg 104:16–23
35. Kurane I, Innis BL, Nimmannitya S, Nisalak A, Meager A, Janus J et al (1991) Activation of T lymphocytes in dengue virus infections. High levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2, and interferon-gamma in sera of children with dengue. J Clin Invest 88:1473–1480
36. Gagnon SJ, Mori M, Kurane I, Green S, Vaughn DW, Kalayanarooj S et al (2002) Cytokine gene expression and protein production in peripheral blood mononuclear cells of children with acute dengue virus infections. J Med Virol 67:41–46
37. Mendonça VR, Souza LC, Garcia GC, Magalhães BM, Lacerda MV, Andrade BB et al (2014) DDX39B (BAT1), TNF and IL6 gene polymorphisms and association with clinical outcomes of patients with *Plasmodium vivax* malaria. Malar J 13:278
38. Rachman A, Rinaldi I (2006) Coagulopathy in dengue infection and the role of interleukin-6. Acta Med Indones 38:105–108
39. Huang Y-H, Lei H-Y, Liu H-S, Lin Y-S, Chen S-H, Liu C-C et al (2003) Tissue plasminogen activator induced by dengue virus infection of human endothelial cells. J Med Virol 70:610–616
40. Mendonça VRR, Queiroz ATL, Lopes FM, Andrade BB, Barral-Netto M (2013) Networking the host immune response in *Plasmodium vivax* malaria. Malar J 12:69
41. Perkins DJ, Weinberg JB, Kremsner PG (2000) Reduced interleukin-12 and transforming growth factor-b1 in severe childhood malaria: relationship of cytokine balance with disease severity. J Infect Dis 182:988–992
42. Luty AJF, Perkins DJ, Lell B, Schmidt-ott R, Lehman LG, Luckner D et al (2000) Low interleukin-12 activity in severe Plasmodium falciparum malaria. Infect Immun 68:3909–3915
43. Doolan DL, Hoffman SL (1999) IL-12 and NK cells are required for antigen-specific adaptive immunity against malaria initiated by CD8+T cells in the Plasmodium yoelii model. J Immunol 163:884–892
44. Marquet S, Doumbo O, Cabantous S, Poudiougou B, Argiro L, Safeukui I et al (2008) A functional promoter variant in IL12B predisposes to cerebral malaria. Hum Mol Genet 17:2190–2195
45. Chaturvedi UC, Agarwal R, Elbishbishi EA, Mustafa AS (2000) Cytokine cascade in dengue hemorrhagic fever: implications for pathogenesis. FEMS Immunol Med Microbiol 28:183–188
46. Basu A, Chaturvedi UC (2008) Vascular endothelium: the battlefield of dengue viruses. FEMS Immunol Med Microbiol 53:287–299
47. de-Oliveira-Pinto LM, Gandini M, Freitas LP, Siqueira MM, Marinho CF, Setúbal S et al (2012) Profile of circulating levels of IL-1Ra, CXCL10/IP-10, CCL4/MIP-1β and CCL2/MCP-1 in dengue fever and parvovirosis. Mem Inst Oswaldo Cruz 107:48–56
48. Berrios V, Kurane I, Ennis FA (1996) Immunomodulatory effects of IL-7 on dengue virus-specific cytotoxic CD4+ T cell clones. Immunol Invest 25:231–240
49. Andrade BB, Reis-Filho A, Souza-Neto SM, Raffaele-Netto I, Camargo LMA, Barral A et al (2010) Plasma superoxide dismutase-1 as a surrogate marker of vivax malaria severity. PLoS Negl Trop Dis 4:e650
50. Hottz ED, Medeiros-de-Moraes IM, Vieira-de-Abreu A, de Assis EF, Vals-de-Souza R, Castro-Faria-Neto HC et al (2014) Platelet activation and apoptosis modulate monocyte inflammatory responses in dengue. J Immunol 193:1864–1872
51. Villar-Centeno LA, Díaz-Quijano FA, Martínez-Vega RA (2008) Biochemical alterations as markers of dengue hemorrhagic fever. Am J Trop Med Hyg 78:370–374

5.7 MANUSCRITO VII

Immunoregulation in human malaria: the challenge of understanding asymptomatic malaria.

Esta revisão descreve o estado da arte dos fatores imunoreguladores humanos relacionados à malária assintomática.

Resumo dos resultados: Ambas respostas imunes, inata e adaptativa, parecem ter papel proeminente na malária assintomática. As atividades das células Treg (com produção de IL-10 e TGF-β) e células B (com ampla resposta de anticorpos) são importantes nesta condição. Além disso, moléculas envolvidas na via de detoxificação do heme livre (como a Hp e HO-1) e no metabolismo do ferro (ferritina e ativação da JNK) tem emergido, recentemente, como biomarcadores confiáveis, e estão ajudando a desvender a imunopatogênese da malária assintomática.

Este trabalho foi publicado no periódico Memórias do Instituto Oswaldo Cruz.

Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. *110*(8): 945-955, December 2015 945

Immunoregulation in human malaria: the challenge of understanding asymptomatic infection

Vitor R de Mendonça[1], Manoel Barral-Netto[1,2]/+

[1]Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, BA, Brasil
[2]Universidade Federal da Bahia, Faculdade de Medicina, Salvador, BA, Brasil

Asymptomatic Plasmodium *infection carriers represent a major threat to malaria control worldwide as they are silent natural reservoirs and do not seek medical care. There are no standard criteria for asymptomatic* Plasmodium *infection; therefore, its diagnosis relies on the presence of the parasite during a specific period of symptomless infection. The antiparasitic immune response can result in reduced* Plasmodium *sp. load with control of disease manifestations, which leads to asymptomatic infection. Both the innate and adaptive immune responses seem to play major roles in asymptomatic* Plasmodium *infection; T regulatory cell activity (through the production of interleukin-10 and transforming growth factor-β) and B-cells (with a broad antibody response) both play prominent roles. Furthermore, molecules involved in the haem detoxification pathway (such as haptoglobin and haeme oxygenase-1) and iron metabolism (ferritin and activated c-Jun N-terminal kinase) have emerged in recent years as potential biomarkers and thus are helping to unravel the immune response underlying asymptomatic* Plasmodium *infection. The acquisition of large data sets and the use of robust statistical tools, including network analysis, associated with well-designed malaria studies will likely help elucidate the immune mechanisms responsible for asymptomatic infection.*

Key words: asymptomatic infection - immune response - biomarkers - networks

It is estimated that two-three billion people are at risk of contracting malaria, and nearly one million people die from this disease each year (WHO 2014). The spectrum of malarial disease can range from severe complications to a mild symptomatic infection to an asymptomatic carrier infection. Such distinct manifestations result from a combination of factors, including parasite virulence, host susceptibility, host immune response, disease tolerance mechanisms, and environmental factors (Andrade & Barral-Netto 2011, Medzhitov et al. 2012).

Although there is no standard definition of asymptomatic plasmodial infection (API), individuals with API harbour the parasite as evidenced by positive parasitaemia. However, these individuals do not develop any symptoms during a defined period of time (Andrade & Barral-Netto 2011, Lindblade et al. 2013). API is an significant obstacle to malaria eradication efforts and represents a serious healthcare problem for the following reasons: (i) serve as parasite reservoirs, which allow malarial disease to be maintained within a population over time as they can still transmit *Plasmodium* sp. to uninfected persons (Gouagna et al. 2004, Alves et al. 2005, Schneider et al. 2007, White 2008), (ii) asymptomatic carriers represent a serious risk to blood bank safety as API carriers can transmit malaria through blood transfusions (Najem & Sulzer 2003, Fugikaha et al. 2007, Scuracchio et al. 2011, Anthony et al. 2013, Brouwer et al. 2013), and (iii) human immunodeficiency virus (HIV)-infected individuals with API sometimes exhibit increased viral load, which may enhance HIV transmission and accelerate disease progression and severity in endemic countries (Verhoeff et al. 1999, Whitworth et al. 2000, French et al. 2001, Kublin et al. 2005).

API can be attributed to several factors, including differences among *Plasmodium* sp. and host protective mechanisms. API is frequently associated with older people living in endemic areas as they are likely to have greater exposure to malaria and its vector in endemic settings over time, thus acquiring a partial immunity (Andrade et al. 2009, Ladeia-Andrade et al. 2009, Mendonça et al. 2013). In the same context, individuals who have had several previous episodes of symptomatic malaria are more likely to become asymptomatic carriers upon *Plasmodium* sp. infection (Andrade et al. 2009, Barbosa et al. 2014). Therefore, the immune response underlying asymptomatic infection still needs to be elucidated.

Individuals from endemic regions can acquire partial immunity to malarial parasites, and antidisease immunity may prevent the development of clinical symptoms of disease despite the presence or the number of parasites. Antiparasitic immunity (after a certain age) against *Plasmodium* sp. suppresses parasite load (Day & Marsh 1991, Trape et al. 1994, Daubersies et al. 1996). The immune response in API is often described as disease resistance, which is associated with a reduction in pathogen burden; therefore, this protective mechanism reduces tissue damage and immunopathology related to malarial infection (Medzhitov et al. 2012). In contrast, some individuals can control disease manifestation despite not being able

doi: 10.1590/0074-02760150241
Financial support: FIOCRUZ (407734/2012-0), CNPq (478527/2013-6)
MB-N is a senior investigator from CNPq.
+ Corresponding author: mbarral@bahia.fiocruz.br
Received 29 June 2015
Accepted 23 October 2015

to reduce levels of parasitaemia; this phenomenon is described as disease tolerance (Medzhitov et al. 2012).

Immunity to malaria does not necessarily prevent infection; however, it does limit parasite density and symptoms (Tran et al. 2013). API individuals can remain infected for long periods even though asymptomatic subjects can develop symptomatic disease if they have a dysregulated immune response (Barbosa et al. 2014). Several studies have reported very low parasitaemia in individuals with API (Perkins et al. 2005, Minigo et al. 2009, Andrade et al. 2010b, Villasis et al. 2012), and many of them exhibited subpatent infections (i.e., infections undetected by microscopy) (Barbosa et al. 2014). Asymptomatic carriers who are not diagnosed with conventional malaria are a major challenge for malaria eradication in low-endemicity settings (Bousema et al. 2014). Taken together, these data illustrate the interaction between malarial immunity, parasitaemia, exposure, and malaria outcomes in endemic areas (Fig. 1).

The immune system seems to play a major role in malaria outcomes, and our object herein is to uncover the partial protective immune response to infection in API to unravel the mechanisms of disease resistance. Here, we review both innate and adaptive immune responses to *Plasmodium* infection as well as new approaches to understand API immunity.

Although not the main focus of this review, it is important to highlight that pathogen-related infections can modulate the immune response of individuals with malaria. In this context, asymptomatic infections have been reported to be composed of multiple genetically distinct *Plasmodium* sp. clones; multiclonal infections may be a marker of immunity and confer protection against malaria by inducing a broader immune response and tolerance to infection (Ntoumi et al. 1995, Felger et al. 1999, Smith et al. 1999, Rono et al. 2013). Regarding others pathogens, hepatitis B co-infection has been associated with *Plasmodium vivax* asymptomatic infection and may also boost the protective immune response (Andrade et al. 2011). Additionally, individuals co-infected with *P. vivax* and hepatitis B virus (HBV) have an increased HBV viraemia yet a decreased malaria parasitaemia (Andrade et al. 2011). These patients also have lower levels of pro-inflammatory tumour necrosis factor (TNF) and a lower interferon (IFN)-γ/interleukin (IL)-10 ratio with higher levels of regulatory IL-10 (Andrade et al. 2011). Pre-existent filarial infection also seems to attenuate immune responses associated with severe *Plasmodium falciparum* malaria and protects against anaemia (Dolo et al. 2012). Co-infections with *Ascaris lumbricoides* or *Schistosoma hematobium* exhibit a trend towards a protective effect, whereas infections with hookworm or *Schistosoma mansoni* lead to aggravation of pathology and a higher incidence of malaria (Adegnika & Kremsner 2012, Lemaitre et al. 2014).

Haemoglobinopathies, including haemoglobin S (HbS), haemoglobin C (HbC), and α-thalassaemia, have been associated with protection from malaria (Mendonça et al. 2012a). API children with HbS and persistently positive smears exhibit a reduced median time for conversion to smear-negative responses (spontaneous clearance) than

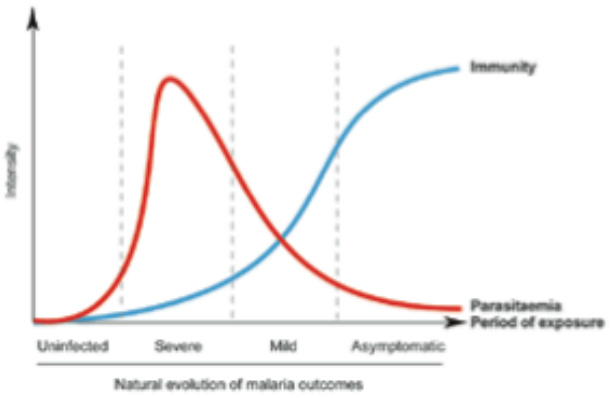


Fig. 1: understanding the natural evolution of malaria outcomes by parasitaemia, immunity, and period of exposure in endemic areas. In endemic settings, the natural evolution of malaria is initiated when uninfected individuals become infected for the first time, usually children who then develop a severe form of the illness. It is known that subjects with severe malaria have high parasitaemias and overall low protective immunity against malaria. In subsequent malarial infections, individuals initiate a more robust immune response against the parasites and exhibit lower levels of parasitaemia and milder forms of this disease. After many years of exposure to malaria and its vector, older people become resistant to malaria by exhibiting higher levels of antiparasitic immunity. Adapted from Andrade and Barral-Netto (2011).

do children without the haemoglobinopathy (Billo et al. 2012). Mechanisms by which haemoglobinopathies may attenuate the pathogenesis of malaria caused by *P. falciparum* include modulation of the inflammatory response and enhancement of cell-mediated and humoural immune responses through pathways that may include haeme oxygenase-1 (HO-1), reduced levels of cerebral chemokines, increased levels of nitric oxide, and higher IgG seroreactivity to *P. falciparum* antigens (Taylor et al. 2013). Other host erythrocyte polymorphisms also seem to influence the susceptibility to malaria. It has been demonstrated that α+-thalassaemia (Oppenheimer et al. 1984, Enevold et al. 2007), southeast Asian ovalocytosis (Cattani et al. 1987, Foo et al. 1992), glucose-6-phosphate dehydrogenase (Mombo et al. 2003), and blood group O polymorphisms (Facer & Brown 1979, Martin et al. 1979, Shimizu et al. 2005) are associated with protection from malaria by reducing parasitic densities.

Innate immunity

It has been reported that neutrophil antibody-dependent respiratory burst (ADRB) activity is correlated with acquired disease resistance to malaria in endemic regions (Joos et al. 2010). In this study, individuals with high ADRB indexes were 17-fold less susceptible to malaria attacks than those without high ADRB activity, and this ADRB activity was dependent on intact merozoites and IgG opsonins but not on parasitized erythrocytes or complement (Joos et al. 2010). Interestingly, the production of reactive oxygen species (ROS) by neutrophilic ADRB in response to *P. falciparum* an-

tigen-specific IgGs was extracellular and indicated a key role for CD32/FcγRII; however, the production of ROS in response to whole merozoites was almost completely within the cell, suggesting that the underlying mechanism was phagocytosis (Kapelski et al. 2014). The innate response to infected red blood cells (RBC) is also related to the functional activity of monocytes (MO) through their phagocytic activity, parasite killing through antibody-dependent cellular inhibition (ADCI), and supplying of peripheral tissues with macrophage and dendritic cells (DCs) (Chimma et al. 2009). Further, individuals with the $CD14^{hi}CCR2^{+}CX3CR1^{+}$ MO subset and the highest mean levels of ADCI activity had lower blood parasitaemia levels, suggesting an antiparasitic activity associated with protection against malaria (Chimma et al. 2009). The induction and maintenance of B and T-cell responses requires functional DCs; these cells also have an important role in malaria immunity, and it was recently described that DCs from individuals with asymptomatic *Plasmodium* infection have higher expression of human leukocyte antigen-DR, which is required for antigen presentation (Kho et al. 2015). In a similar manner in a rodent model, DCs from nonlethal infections were fully functional and capable of secreting cytokines and stimulating T-cells compared to DCs from lethal infections, suggesting a major role for this cell in disease outcome and immunity (Wykes et al. 2007). Cells of the innate immune response are the first line of human defence against pathogens and may be important in control of the parasitaemia underlying cases of API.

Adaptive immunity

The innate immune system also helps direct the response of adaptive immune cells (B and T-cells) in recognising and binding diverse antigens through a repertoire of cell surface receptors (Palm & Medzhitov 2009). It has been demonstrated that $CD4^{+}$ and $CD8^{+}$ T-cells are important for malarial immunity in humans as well as in mouse models (Nussenzweig et al. 1967, Clyde et al. 1973, Schofield et al. 1987, Romero et al. 1989, Rodrigues et al. 1993, Tsuji et al. 1998, Hoffman & Doolan 2000, Stephens et al. 2005, Overstreet et al. 2008, Schmidt et al. 2008, Roestenberg et al. 2009, 2011, Stephens & Langhorne 2010, Friesen et al. 2010). In a clinical trial of the RTS,S/AS01E antimalarial vaccine, $CD4^{+}$ T-cell production of TNF, with or without IFN-γ, was a potential immunologic correlate of protection against disease in individuals from an endemic area (Olotu et al. 2011). $CD4^{+}$ cells from individuals with fewer previous episodes of malaria were more inflammatory and had greater TNF production, whereas responses from $CD4^{+}$ T-cells from subjects with more frequent previous episodes of malaria were more typical of regulatory T-cells in that they produced IL-10 (Jagannathan et al. 2014). In this report, the absence of pro-inflammatory $CD4^{+}$ T-cells producing TNF was associated with asymptomatic infection (Jagannathan et al. 2014). Thus, it suggests that IL-10 production by T-helper 1 T-cells may help prevent immunopathology by dampening the pro-inflammatory response (TNF) and preventing the development of clinical disease (Jagannathan et al. 2014).

T regulatory (Treg) cells ($CD4^{+}CD25^{+}FOXP3^{+}$) appear to mediate their effects by direct cell contact or by induction of the regulatory cytokines IL-10 or transforming growth factor (TGF)-β (Thornton & Shevach 2000, Powrie et al. 2003). Treg cells are induced following *P. falciparum* and *P. vivax* infection and are associated with a burst of TGF-β production and decreased pro-inflammatory cytokine production (Walther et al. 2005, Gonçalves et al. 2010). Nevertheless, exposed asymptomatic controls (with or without parasitaemia) in a malaria-endemic region of Indonesia had a lower frequency of Treg cells ($CD4^{+}CD25^{+}Foxp3^{+}CD127^{lo}$) than did patients with uncomplicated and severe malaria, suggesting a role for Treg reduction in malaria protection (Minigo et al. 2009). Intriguingly, increased expression of TNFRII, a marker of Treg activation, was found in Treg cells from API subjects when compared with uninfected individuals, a feature that might be important for survival of the parasites in asymptomatic carriers; however, TNFRII expression was not measured in patients with mild or severe malaria (Wammes et al. 2013). Congolese children with asymptomatic infection have a higher prevalence of polymorphisms in regulatory genes (*STAT6* and *IL10RA*), which may influence Treg cells and malaria protection (Koukouikila-Koussounda et al. 2013).

The humoural response is also important for malaria protection because passive transfer of IgG from immune African adults to children and nonimmune adults with acute malaria rapidly reduces parasitaemia and abrogates fever (Cohen et al. 1961, Sabchareon et al. 1991). Not all exposure to malaria results in the generation of memory B-cells (MBCs) and IgG antibodies against *P. falciparum* are short-lived and fail to boost upon re-infection. Thus, immunological memory is a challenge in many vaccine trials (Dorfman et al. 2005, Bejon et al. 2006). Previous studies have described an atypical MBC population (characterised by the expression of FcRL4 and hyporesponsiveness) that is expanded in *P. falciparum*-exposed adults and children from Mali when compared with healthy United States of America controls, suggesting that this atypical population may contribute to the delayed acquisition and short-lived nature of malarial B-cell immunity (Weiss et al. 2009). Recently, it was described that atypical MBCs appear to differentiate from classical MBCs, and express a repertoire of inhibitory receptors and a deficient B-cell receptor signalling, which leads to impaired B-cell proliferation, cytokine production, and antibody secretion (Portugal et al. 2015). Other B-cells subtypes also seem to influence malaria resistance as Portugal et al. (2012) demonstrated that the percentage of activated MBCs and plasma cells was higher in the resistant Fulani ethnic group compared to those in the susceptible Dogon ethnic group, suggesting a role for B-cells in the protective immunity observed in the Fulani individuals. Individuals with asymptomatic infection tend to have higher titres of *P. falciparum* antigen-specific IgG than do individuals with other malaria outcomes. This higher response has been described as specific to several antigens, such as *P. falciparum* rifin on the surface of RBCs, recombinant protein fragments of *P. falciparum* rhoptry-associated

protein-1, *P. falciparum* merozoite protein (C-terminal 10 kD), *P. falciparum* CLAG 9 (composed of 3 subunits named RhopH1, RhopH2, and RhopH3), and malaria-infected erythrocyte variant surface antigens, including *P. falciparum* erythrocyte membrane protein 1, *P. falciparum* merozoite surface protein 1 3D7 (MSP142), *P. falciparum* VarO rosetting variant, and *P. falciparum* erythrocyte binding-like and reticulocyte binding-like proteins (Alifrangis et al. 1999, Braga et al. 2002, Abdel-Latif et al. 2003, Kinyanjui et al. 2004, Villasis et al. 2012, Costa et al. 2013, Moormann et al. 2013, Sagna et al. 2013). Further, high antibody levels against glycosylphosphatidylinositols, the anchor molecules of some membrane proteins of *Plasmodium* species, is also observed more frequently in children with asymptomatic infections than in children with symptomatic infections in The Gambia (de Souza et al. 2002). Asymptomatic malaria carriers were also associated with high antibody levels against human brain antigens and *Escherichia coli* proteins as a result of polyclonal immunoglobin reactivity (Fesel et al. 2005). Furthermore, our group described that higher titre of IgG antibody against *Anopheles darlingi* mosquito saliva is also associated with immunity in asymptomatic *P. vivax* individuals from the Brazilian Amazon Region as a result of higher exposure to the malaria vector (Andrade et al. 2009). The intense production of antibodies in asymptomatic malaria carriers represents an active immune response and highlights the role of the humoural immune response in mediating disease resistance.

Biomarkers

A biomarker is any parameter that can be used as an indicator of a particular disease state or other physiological state and can be generally classified as either biomarkers for diagnosis or for disease severity (Andrade & Barral-Netto 2011). In the context of API, biomarkers can help investigators understand disease pathology by measuring important parameters in various immune pathways and may also be useful as markers of prognosis in either clinical or silent infection after *Plasmodium* sp. exposure (Laishram et al. 2012). In recent years, our group and others have been searching for human genetic factors and plasma measures related to the immune response associated with asymptomatic infection. However, none of these factors was sufficiently powerful to be a prognostic surrogate marker of clinical protection or disease susceptibility (Andrade & Barral-Netto 2011, Mendonça et al. 2012b).

Laboratory measures are commonly used in medical practice as organ dysfunction parameters; individuals with asymptomatic *P. vivax* malaria have lower levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), indirect bilirubin, and serum creatinine as well as higher levels of Hb than do individuals with mild or severe symptomatic *P. vivax* malaria (Andrade et al. 2010b). TNF is a pro-inflammatory cytokine that has attracted special interest because of its ambiguous activity in host defence and in the pathogenesis of cerebral malaria and other severe complications (Kwiatkowski 2000). An increased TNF concentration is associated with symptoms of mild malarial pathogenesis (i.e., fever) as well as severe forms of infection, such as cerebral malaria (Kwiatkowski et al. 1990, Karunaweera et al. 1992). However, TNF-α has also been associated with the presence of potent antiparasitic activity as persistently elevated levels of this cytokine lead to rapid improvement of fever and reduction of parasitaemia (Mordmüller et al. 1997, Depinay et al. 2011). It is also noteworthy that patients with asymptomatic *P. vivax* malaria have lower levels of pro-inflammatory TNF and IFN-γ and higher levels of IL-10, a trend which is proportional to disease severity (asymptomatic, mild, and severe) and which may explain the immunological control of clinical disease. However, parasite burden control may involve a more complex host response in addition to the moderation of TNF levels (Andrade et al. 2010b, Mendonça et al. 2013). In another setting in the Brazilian Amazon, it was found that asymptomatic carriers of low *P. vivax* parasitaemias also had lower levels of TNF and IFN-γ than did symptomatic *P. falciparum* or *P. vivax* subjects (Gonçalves et al. 2012). Furthermore, certain combinations of genotypes in inflammatory-related genes (*DDX39B*, *TNF* and *IL6*) are associated with a decreased risk of mild malaria compared to asymptomatic infection by reducing plasma levels of IL-6 and TNF (Mendonça et al. 2014).

The immune and organ dysfunction response during malaria may be a result, at least in part, of the harmful effects of free haem in the human host (Gozzelino et al. 2010). During parasite-induced intravascular haemolysis, great amounts of Hb are liberated; in the presence of superoxide and other ROS, Hb releases its haem prosthetic group (Bunn & Jandl 1968, Hebbel et al. 1988, Pamplona et al. 2007, Ferreira et al. 2008). Free haem is a harmful molecule and can cause cytotoxicity, inflammation, oxidative stress, and even cell death (Ferreira et al. 2008, Gozzelino & Soares 2011). Free haem levels exhibit a linear increase according to disease severity in asymptomatic *P. vivax*-infected subjects with the lowest haem plasma concentrations (Andrade et al. 2010a). In addition, haem is also elevated with malaria severity by *P. falciparum*, especially for cerebral malaria and acute renal failure subjects (Dalko et al. 2015). In addition to enhancing pro-inflammatory mechanisms, free haem during *P. vivax* malaria also impairs prostaglandin E2 (PGE2) and TGF-β production through superoxide dismutase (SOD)-1-dependent mechanism (Andrade et al. 2010a). SOD-1 is also elevated proportionally with disease severity in malaria patients and is useful for distinguishing mild and asymptomatic *P. vivax* cases by ROC curve analysis (Andrade et al. 2010c). In addition, asymptomatic carriers have higher concentrations of regulatory cytokines such as TGF-β and PGE2 compared with mild and severe *P. vivax* patients, and TGF-β and PGE2 are negatively correlated with SOD-1, which may be an additional defence mechanism against disease manifestation (Andrade et al. 2010a). In *P. falciparum* malaria, bicyclo-PGE2 is also elevated in asymptomatic patients compared with patients who have symptomatic disease (Perkins et al. 2005).

Over time, the human host has evolved protective mechanisms against the deleterious effects of free haem in the circulation. When Hb is released from ruptured RBC upon *Plasmodium* sp. infection, it is scavenged by haptoglobin (Hp) and prevents the release of haem. The

complex Hp-Hb is recognised by CD163 on the macrophage and hepatocyte surfaces in the spleen and liver, respectively (Philippidis et al. 2004, Quaye 2008). Free haem can also be scavenged by haemopexin, albumin, α1-microglobulin, and high and low-density lipoproteins (Bunn & Jandl 1966, Miller & Shaklai 1999, Paoli et al. 1999, Allhorn et al. 2002, Fasano et al. 2007, Tolosano et al. 2010). Different Hp phenotypes are known to have different binding affinities for cell-free Hb (Hp1.1>Hp1.2>Hp2.2) and CD163 (Hp2.2>Hp1.2>Hp1.1) (Kristiansen et al. 2001). Our group has reported that individuals with the *Hp2* allele are more likely to have symptomatic *P. vivax* malaria, and this group also has higher levels of Hp when compared with those of patients with asymptomatic infection. This probably represents a compensatory mechanism against the low binding affinity of *Hp2* to free Hb (Mendonça et al. 2012b). The Hp2.2 phenotype has also been associated with a higher susceptibility to *P. falciparum* infection in the Dogon ethnic group living in Mali (Perdijk et al. 2013). Furthermore, soluble CD163 (sCD163) (marker of receptor activation) is also lower in asymptomatic patients when compared with that in symptomatic subjects, and a cut-off value of sCD163 may be used to distinguish between symptomatic and disease-free individuals (Mendonça et al. 2012b). In Mali, sCD163 was increased in *P. falciparum* infected individuals compared to uninfected subjects (Perdijk et al. 2013). Inside the cell, haem is degraded by HO-1 to produce carbon monoxide (CO), labile iron, and biliverdin. In murine models, HO-1 affords protection against cerebral malaria by reducing neuroinflammation (including $CD8^+$ T-cell brain sequestration), and exposure to CO may reduce severe complications (Pamplona et al. 2007). HO-1 also seems to be one of the mechanisms by which sickle cell disease confers protection against experimental malaria (Ferreira et al. 2011). HO-1 plasma levels are higher in symptomatic cases (as compared to asymptomatic individuals) as a regulatory defence, and a microsatellite polymorphism (GT)n in *HMOX1* regulates the expression of this enzyme (Mendonça et al. 2012b). In addition, high HO-1 levels and this microsatellite polymorphism were associated with severe malaria, including death, in another study (Walther et al. 2012). However, other studies also have demonstrated conflicting results and no association between this *HMOX1* microsatellite polymorphism and malaria severity (Kuesap et al. 2010, Hansson et al. 2015).

Iron is produced by haem catabolism and also obtained by dietary uptake; this metal is necessary for complete *Plasmodium* development (Gozzelino et al. 2010). However, intracellular labile iron is dangerous because it converts to a free radical unless it is scavenged by ferritin, which acts as a vital antioxidant molecule in several experimental models (Balla et al. 1992, Cozzi et al. 2000, Berberat et al. 2003). Ferritin serum levels are decreased and associated with anaemia in a population from the Brazilian Amazon exposed to *P. vivax* malaria; symptomatic individuals from this group infected with *P. vivax* have lower levels of ferritin, which are directly proportional to the hepatic damage score (Cardoso et al. 1994, Gozzelino et al. 2012). It has been reported that ferritin promotes disease resistance to malaria by preventing labile intracellular iron from sustaining pro-apoptotic c-Jun N-terminal kinase activation, and this tolerance requires the expression of HO-1 (Gozzelino et al. 2012). Interestingly, malarial tolerance mediated by ferritin production is independent of the parasitaemia rate and represents a host defence strategy to limit the fitness costs of infection irrespective of pathogen burden (Medzhitov et al. 2012).

New approaches to understanding asymptomatic infection

In recent years, large amounts of data have become available as a result of the progress in technological methods, such as multiplex measurements, genome-wide genotyping, microarrays, RNAseq, and multicolour flow cytometry (Tran et al. 2012). Genome-wide studies allowed the discovery of important *loci* related to malaria resistance and low parasitaemia. Linkage of asymptomatic parasitaemia to 5q31-q33 has been reported in humans (Rihet et al. 1998, Timmann et al. 2007) and, recently, chromosomes 6p21.3 and 17p12 were correlated with resistance in individuals from Burkina Faso (Brisebarre et al. 2014). Equally important, the field of engineered humoural immunity (with the production of human monoclonal antibodies) has allowed a better understanding of the malaria immune response by facilitating several laboratory methods (i.e., multiparameter flow cytometry).

To understand this large volume of information, new approaches for data analysis have become more widespread and multivariate (clusters, principal component analysis, etc.), artificial neural, Bayesian, and network analysis methods are some tools that can be used to characterise a molecular signature of resistance or susceptibility to malaria (Jayavanth & Singh 2003, Kiang et al. 2006, da Cunha et al. 2010, Bachtiar et al. 2013). Many studies have attempted to identify molecular signatures associated with severe *P. falciparum* malaria, but few have focused on the mechanisms behind asymptomatic *Plasmodium* infection (Timmann et al. 2007, 2012, Jallow et al. 2009, Milet et al. 2010). Using a network approach, our group recently described the interactions among cytokines, chemokines and other inflammatory proteins associated with different *P. vivax* malaria outcomes (Mendonça et al. 2013). Network analysis allows a better understanding of the inflammatory profile from different malaria groups by allowing easy visualisation of interactions between several markers and identification of patterns of association that may indicate susceptibility or disease tolerance signatures. Using network analysis, it has been demonstrated that patients with asymptomatic *P. vivax* malaria have an overall reduction in pro-inflammatory cytokines (TNF, IFN-γ, IL-6) and markers of tissue damage (ALT, AST, creatinine, bilirubin, and others) and augmented levels of regulatory cytokines (TGF-β and IL-10) when compared with those of the symptomatic groups (mild and severe malaria) (Mendonça et al. 2013). Furthermore, IL-4 had the highest number of interactions between all the markers in the asymptomatic group, suggesting a possible role for this cytokine in mediating *P. vivax* malaria tolerance (Mendonça et al. 2013). Others studies have also used the same network

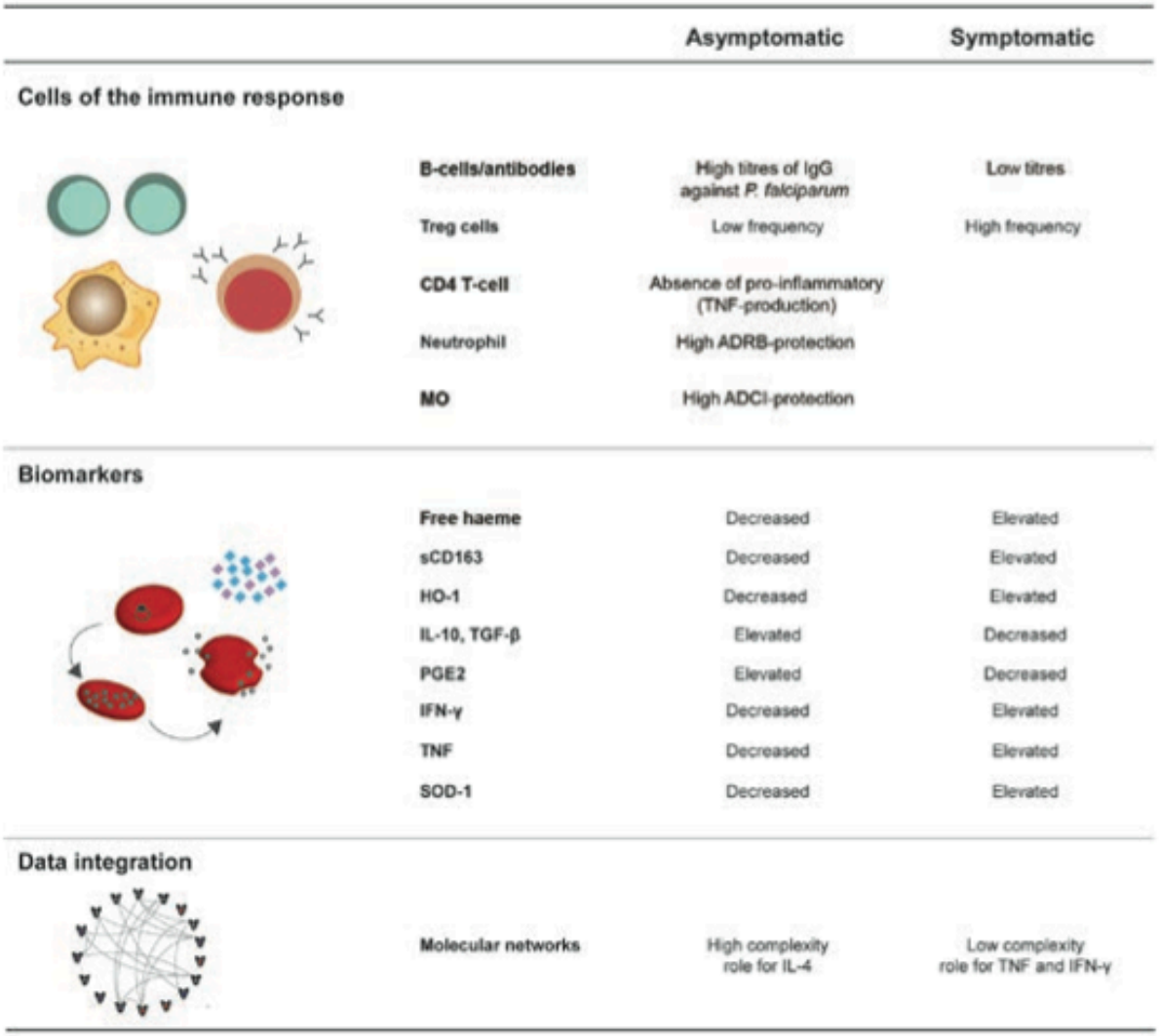


Fig. 2: the immune response underlying asymptomatic infection. Aspects of the immune response of asymptomatic malaria carriers were compared to symptomatic patients. This response was didactically divided into immune cells including T regulatory (Treg) cells, $CD4^+$ T-cells, B-cells, neutrophils, and monocytes (MOs) and biomarkers related to inflammation [interleukin (IL)-10, transforming growth factor (TGF)-β, prostaglandin E2 (PGE2), interferon (IFN)-γ, and tumour necrosis factor (TNF) and the haeme pathway [haeme, soluble CD163 (sCD163), haeme oxygenase-1 (HO-1), and superoxide dismutase (SOD)-1]. Additionally, molecular networks in the context of asymptomatic infection illustrate the use of methods of data integration in immunology. ADCI: antibody-dependent cellular inhibition; ADRB: antibody-dependent respiratory burst.

analysis for placental malaria and malarial anaemia, but none analysed asymptomatic infection (Ong'echa et al. 2011, Sikora et al. 2011). In this context, cohort studies with a large sample size and an extensive bioinformatics approach are highly necessary to better understand the interactions among the immune response pathways associated with asymptomatic infection tolerance.

Concluding remarks

It is noteworthy that API is related to clinical disease tolerance (i.e., absence of symptomatology) but is not associated with immunity and inflammatory tolerance. Asymptomatic *P. vivax* infection is an active and acquired state, and it can control parasitaemia and limit organ dysfunction by an as yet poorly understood immune mechanism. Asymptomatic individuals carrying the parasite are natural reservoirs representing a challenge for malaria eradication, primarily in low and moderate-endemic countries. The use of mass drug administration or mass screening and treatment schemes is controversial (Tada et al. 2012). Overall, biomarkers related to the haem pathway and iron metabolism have emerged in recent years as potential clues to unravel the immune response of API. Despite this progress, there is no reliable marker of prognosis in API. Immune cells, especially Tregs and B-cells, seem to play an important role in protection from disease manifestation. Furthermore, it has been observed that the immune response in individuals with asymptomatic infection is usually associated with a lower pro-inflammatory and a higher regulatory production of biomarkers and host genetic alterations that may contribute to malaria tolerance. Nevertheless,

the acquisition of large-scale biological data along with the use of robust bioinformatics tools, including a network approach, will help investigators to understand the immune response behind asymptomatic infection. The major topics described here are summarised in Fig. 2. Longitudinal studies of sequential episodes of malaria in the same individual are necessary to better understand the immune response of individuals with API who are able to clear their parasitaemia compared with those who are more likely to have a symptomatic disease or remain symptomless despite the presence of *Plasmodium* sp. With this understanding, better medical management of API carriers, the development of malarial vaccines, and strategies for malaria eradication will be facilitated.

REFERENCES

Abdel-Latif MS, Dietz K, Issifou S, Kremsner PG, Klinkert M-Q 2003. Antibodies to *Plasmodium falciparum* rifin proteins are associated with rapid parasite clearance and asymptomatic infections. *Infect Immun 71*: 6229-6233.

Adegnika AA, Kremsner PG 2012. Epidemiology of malaria and helminth interaction: a review from 2001 to 2011. *Curr Opin HIV AIDS 7*: 221-224.

Alifrangis M, Lemnge MM, Moon R, Theisen M, Bygbjerg I, Ridley RG, Jakobsen PH 1999. IgG reactivities against recombinant rhoptry-associated protein-1 (rRAP-1) are associated with mixed *Plasmodium* infections and protection against disease in Tanzanian children. *Parasitology 119*: 337-342.

Allhorn M, Berggård T, Nordberg J, Olsson ML, Akerström B 2002. Processing of the lipocalin alpha(1)-microglobulin by hemoglobin induces heme-binding and heme-degradation properties. *Blood 99*: 1894-1901.

Alves FP, Gil LHS, Marrelli MT, Ribolla PEM, Camargo EP, da Silva LHP 2005. Asymptomatic carriers of *Plasmodium* spp as infection source for malaria vector mosquitoes in the Brazilian Amazon. *J Med Entomol 42*: 777-779.

Andrade BB, Araújo-Santos T, Luz NF, Khouri R, Bozza MT, Camargo LMA, Barral A, Borges VM, Barral-Netto M 2010a. Heme impairs prostaglandin E2 and TGF-beta production by human mononuclear cells *via* Cu/Zn superoxide dismutase: insight into the pathogenesis of severe malaria. *J Immunol 185*: 1196-1204.

Andrade BB, Barral-Netto M 2011. Biomarkers for susceptibility to infection and disease severity in human malaria. *Mem Inst Oswaldo Cruz 106* (Suppl. I): 70-78.

Andrade BB, Reis-Filho A, Souza-Neto SM, Clarêncio J, Camargo LMA, Barral A, Barral-Netto M 2010b. Severe *Plasmodium vivax* malaria exhibits marked inflammatory imbalance. *Malar J 9*: 13.

Andrade BB, Reis-Filho A, Souza-Neto SM, Raffaele-Netto I, Camargo LMA, Barral A, Barral-Netto M 2010c. Plasma superoxide dismutase-1 as a surrogate marker of vivax malaria severity. *PLoS Negl Trop Dis 4*: e650.

Andrade BB, Rocha BC, Reis-Filho A, Camargo LMA, Tadei WP, Moreira LA, Barral A, Barral-Netto M 2009. Anti-*Anopheles darlingi* saliva antibodies as marker of *Plasmodium vivax* infection and clinical immunity in the Brazilian Amazon. *Malar J 8*: 121.

Andrade BB, Santos CJN, Camargo LM, Souza-Neto SM, Reis-Filho A, Clarêncio J, Mendonça VRR, Luz NF, Camargo EP, Barral A, Silva AAM, Barral-Netto M 2011. Hepatitis B infection is associated with asymptomatic malaria in the Brazilian Amazon. *PLoS One 6*: e19841.

Anthony CN, Lau Y-L, Sum J-S, Fong M-Y, Ariffin H, Zaw W-L, Jeyajothi I, Mahmud R 2013. Malaysian child infected with *Plasmodium vivax via* blood transfusion: a case report. *Malar J 12*: 308.

Bachtiar LR, Unsworth CP, Newcomb RD 2013. Application of artificial neural networks on mosquito olfactory receptor neurons for an olfactory biosensor. *Conf Proc IEEE Eng Med Biol Soc 2013*: 5390-5393.

Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM 1992. Ferritin: a cytoprotective antioxidant strategem of endothelium. *J Biol Chem 267*: 18148-18153.

Barbosa S, Gozze AB, Lima NF, Batista CL, Bastos M da S, Nicolete VC, Fontoura PS, Gonçalves RM, Viana SAS, Menezes MJ, Scopel KKG, Cavasini CE, Malafronte R dos S, da Silva-Nunes M, Vinetz JM, Castro MC, Ferreira MU 2014. Epidemiology of disappearing *Plasmodium vivax* malaria: a case study in rural Amazonia. *PLoS Negl Trop Dis 8*: e3109.

Bejon P, Mwacharo J, Kai O, Mwangi T, Milligan P, Todryk S, Keating S, Lang T, Lowe B, Gikonyo C, Molyneux C, Fegan G, Gilbert SC, Peshu N, Marsh K, Hill AVS 2006. A Phase 2b randomised trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya. *PLoS Clin Trials 1*: e29.

Berberat PO, Katori M, Kaczmarek E, Anselmo D, Lassman C, Ke B, Shen X, Busuttil RW, Yamashita K, Csizmadia E, Tyagi S, Otterbein LE, Brouard S, Tobiasch E, Bach FH, Kupiec-Weglinski JW, Soares MP 2003. Heavy chain ferritin acts as an antiapoptotic gene that protects livers from ischemia reperfusion injury. *FASEB J 17*: 1724-1726.

Billo MA, Johnson ES, Doumbia SO, Poudiougou B, Sagara I, Diawara SI, Diakité M, Diallo M, Doumbo OK, Tounkara A, Rice J, James MA, Krogstad DJ 2012. Sickle cell trait protects against *Plasmodium falciparum* infection. *Am J Epidemiol 176* (Suppl.): S175-S185.

Bousema T, Okell L, Felger I, Drakeley C 2014. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. *Nat Rev Microbiol 12*: 833-840.

Braga EM, Barros RM, Reis TA, Fontes CJF, Morais CG, Martins MS, Krettli AU 2002. Association of the IgG response to *Plasmodium falciparum* merozoite protein (C-terminal 19 kD) with clinical immunity to malaria in the Brazilian Amazon Region. *Am J Trop Med Hyg 66*: 461-466.

Brisebarre A, Kumulungui B, Sawadogo S, Atkinson A, Garnier S, Fumoux F, Rihet P 2014. A genome scan for *Plasmodium falciparum* malaria identifies quantitative trait *loci* on chromosomes 5q31, 6p21.3, 17p12, and 19p13. *Malar J 13*: 198.

Brouwer EE, van Hellemond JJ, van Genderen PJ, Slot E, van Lieshout L, Visser LG, Wismans PJ 2013. A case report of transfusion-transmitted *Plasmodium malariae* from an asymptomatic non-immune traveller. *Malar J 12*: 439.

Bunn HF, Jandl JH 1966. Exchange of heme among hemoglobin molecules. *Proc Natl Acad Sci USA 56*: 974-978.

Bunn HF, Jandl JH 1968. Exchange of heme among hemoglobins and between hemoglobin and albumin. *J Biol Chem 243*: 465-475.

Cardoso MA, Ferreira MU, Camargo LM, Szarfarc SC 1994. Anaemia, iron deficiency and malaria in a rural community in Brazilian Amazon. *Eur J Clin Nutr 48*: 326-332.

Cattani JA, Gibson FD, Alpers MP, Crane GG 1987. Hereditary ovalocytosis and reduced susceptibility to malaria in Papua New Guinea. *Trans R Soc Trop Med Hyg 81*: 705-709.

Chimma P, Roussilhon C, Sratongno P, Ruangveerayuth R, Pattanapanyasat K, Pérignon J-L, Roberts DJ, Druilhe P 2009. A distinct peripheral blood monocyte phenotype is associated with parasite inhibitory activity in acute uncomplicated *Plasmodium falciparum* malaria. *PLoS Pathog 5*: e1000631.

Clyde DF, Most H, McCarthy VC, Vanderberg JP 1973. Immunization of man against sporozite-induced falciparum malaria. *Am J Med Sci 266*: 169-177.

Cohen S, McGregor IA, Carrington S 1961. Gamma-globulin and acquired immunity to human malaria. *Nature 192*: 733-737.

Costa JDN, Zanchi FB, Rodrigues FLS, Honda ER, Katsuragawa TH, Pereira DB, Taborda RLM, Tada MS, Ferreira RGM, Pereira-da-Silva LH 2013. Cross-reactive anti-PfCLAG9 antibodies in the sera of asymptomatic parasite carriers of *Plasmodium vivax*. *Mem Inst Oswaldo Cruz 108*: 98-105.

Cozzi A, Corsi B, Levi S, Santambrogio P, Albertini A, Arosio P 2000. Overexpression of wild type and mutated human ferritin H-chain in HeLa cells: in vivo role of ferritin ferroxidase activity. *J Biol Chem 275*: 25122-25129.

da Cunha GB, Luitgards-Moura JF, Naves ELM, Andrade AO, Pereira AA, Milagre ST 2010. Use of an artificial neural network to predict the incidence of malaria in the city of Cantá, state of Roraima. *Rev Soc Bras Med Trop 43*: 567-570.

Dalko E, Das B, Herbert F, Fesel C, Pathak S, Tripathy R, Cazenave P-A, Ravindran B, Sharma S, Pied S 2015. Multifaceted roles of heme during severe *Plasmodium falciparum* infections in India. *Infect Immun 83*: 3793-3799.

Daubersies P, Sallenave-Sales S, Magne S, Trape JF, Contamin H, Fandeur T, Rogier C, Mercereau-Puijalon O, Druilhe P 1996. Rapid turnover of *Plasmodium falciparum* populations in asymptomatic individuals living in a high transmission area. *Am J Trop Med Hyg 54*: 18-26.

Day KP, Marsh K 1991. Naturally acquired immunity to *Plasmodium falciparum*. *Immunol Today 12*: A68-71.

de Souza JB, Todd J, Krishegowda G, Gowda DC, Kwiatkowski D, Riley EM 2002. Prevalence and boosting of antibodies to *Plasmodium falciparum* glycosylphosphatidylinositols and evaluation of their association with protection from mild and severe clinical malaria. *Infect Immun 70*: 5045-5051.

Depinay N, Franetich JF, Grüner AC, Mauduit M, Chavatte J-M, Luty AJF, van Gemert G-J, Sauerwein RW, Siksik J-M, Hannoun L, Mazier D, Snounou G, Rénia L 2011. Inhibitory effect of TNF-α on malaria pre-erythrocytic stage development: influence of host hepatocyte/parasite combinations. *PLoS One 6*: e17464.

Dolo H, Coulibaly YI, Dembele B, Konate S, Coulibaly SY, Doumbia SS, Diallo AA, Soumaoro L, Coulibaly ME, Diakite SAS, Guindo A, Fay MP, Metenou S, Nutman TB, Klion AD 2012. Filariasis attenuates anemia and proinflammatory responses associated with clinical malaria: a matched prospective study in children and young adults. *PLoS Negl Trop Dis 6*: e1890.

Dorfman JR, Bejon P, Ndungu FM, Langhorne J, Kortok MM, Lowe BS, Mwangi TW, Williams TN, Marsh K 2005. B-cell memory to 3 *Plasmodium falciparum* blood-stage antigens in a malaria-endemic area. *J Infect Dis 191*: 1623-1630.

Enevold A, Alifrangis M, Sanchez JJ, Carneiro I, Roper C, Børsting C, Lusingu J, Vestergaard LS, Lemnge MM, Morling N, Riley E, Drakeley CJ 2007. Associations between alpha+-thalassemia and *Plasmodium falciparum* malarial infection in northeastern Tanzania. *J Infect Dis 196*: 451-459.

Facer CA, Brown J 1979. ABO blood groups and falciparum malaria. *Trans R Soc Trop Med Hyg 73*: 599-600.

Fasano M, Fanali G, Leboffe L, Ascenzi P 2007. Heme binding to albuminoid proteins is the result of recent evolution. *IUBMB Life 59*: 436-440.

Felger I, Irion A, Steiger S, Beck HP 1999. Genotypes of merozoite surface protein 2 of *Plasmodium falciparum* in Tanzania. *Trans R Soc Trop Med Hyg 93* (Suppl. 1): 3-9.

Ferreira A, Balla J, Jeney V, Balla G, Soares MP 2008. A central role for free heme in the pathogenesis of severe malaria: the missing link? *J Mol Med (Berl) 86*: 1097-1111.

Ferreira A, Marguti I, Bechmann I, Jeney V, Chora A, Palha NR, Rebelo S, Henri A, Beuzard Y, Soares MP 2011. Sickle hemoglobin confers tolerance to *Plasmodium* infection. *Cell 145*: 398-409.

Fesel C, Goulart LF, Silva Neto A, Coelho A, Fontes CJF, Braga EM, Vaz NM 2005. Increased polyclonal immunoglobulin reactivity toward human and bacterial proteins is associated with clinical protection in human *Plasmodium* infection. *Malar J 4*: 5.

Foo LC, Rekhraj V, Chiang GL, Mak JW 1992. Ovalocytosis protects against severe malaria parasitemia in the Malayan aborigines. *Am J Trop Med Hyg 47*: 271-275.

French N, Nakiyingi J, Lugada E, Watera C, Whitworth JA, Gilks CF 2001. Increasing rates of malarial fever with deteriorating immune status in HIV-1-infected Ugandan adults. *AIDS 15*: 899-906.

Friesen J, Silvie O, Putrianti ED, Hafalla JCR, Matuschewski K, Borrmann S 2010. Natural immunization against malaria: causal prophylaxis with antibiotics. *Sci Transl Med 2*: 40ra49.

Fugikaha E, Fornazari PA, Penhalbel R de SR, Lorenzetti A, Maroso RD, Amoras JT, Saraiva AS, Silva RU da, Bonini-Domingos CR, Mattos LC de, Rossit ARB, Cavasini CE, Machado RLD 2007. Molecular screening of *Plasmodium* sp. asymptomatic carriers among transfusion centers from Brazilian Amazon Region. *Rev Inst Med Trop Sao Paulo 49*: 1-4.

Gonçalves RM, Salmazi KC, Santos BAN, Bastos MS, Rocha SC, Boscardin SB, Silber AM, Kallás EG, Ferreira MU, Scopel KKG 2010. $CD4^+CD25^+Foxp3^+$ regulatory T-cells, dendritic cells, and circulating cytokines in uncomplicated malaria: do different parasite species elicit similar host responses? *Infect Immun 78*: 4763-4772.

Gonçalves RM, Scopel KKG, Bastos MS, Ferreira MU 2012. Cytokine balance in human malaria: does *Plasmodium vivax* elicit more inflammatory responses than *Plasmodium falciparum*? *PLoS One 7*: e44394.

Gouagna LC, Ferguson HM, Okech BA, Killeen GF, Kabiru EW, Beier JC, Githure JI, Yan G 2004. *Plasmodium falciparum* malaria disease manifestations in humans and transmission to *Anopheles gambiae*: a field study in Western Kenya. *Parasitology 128*: 235-243.

Gozzelino R, Andrade BB, Larsen R, Luz NF, Vanoaica L, Seixas E, Coutinho A, Cardoso S, Rebelo S, Poli M, Barral-Netto M, Darshan D, Kühn LC, Soares MP 2012. Metabolic adaptation to tissue iron overload confers tolerance to malaria. *Cell Host Microbe 12*: 693-704.

Gozzelino R, Jeney V, Soares MP 2010. Mechanisms of cell protection by heme oxygenase-1. *Annu Rev Pharmacol Toxicol 50*: 323-354.

Gozzelino R, Soares MP 2011. Heme sensitization to TNF-mediated programmed cell death. *Adv Exp Med Biol 691*: 211-219.

Hansson HH, Maretty L, Balle C, Goka BQ, Luzon E, Nkrumah FN, Schousboe ML, Rodrigues OP, Bygbjerg IC, Kurtzhals JAL, Alifrangis M, Hempel C 2015. Polymorphisms in the haem oxygenase-1 promoter are not associated with severity of *Plasmodium falciparum* malaria in Ghanaian children. *Malar J 14*: 153.

Hebbel RP, Morgan WT, Eaton JW, Hedlund BE 1988. Accelerated autoxidation and heme loss due to instability of sickle hemoglobin. *Proc Natl Acad Sci USA 85*: 237-241.

Hoffman SL, Doolan DL 2000. Malaria vaccines-targeting infected hepatocytes. *Nat Med 6*: 1218-1219.

Jagannathan P, Eccles-James I, Bowen K, Nankya F, Auma A, Wamala S, Ebusu C, Muhindo MK, Arinaitwe E, Briggs J, Greenhouse B, Tappero JW, Kamya MR, Dorsey G, Feeney ME 2014. IFNγ/

IL-10 co-producing cells dominate the CD4 response to malaria in highly exposed children. *PLoS Pathog 10*: e1003864.

Jallow M, Teo YY, Small KS, Rockett KA, Deloukas P, Clark TG, Kivinen K, Bojang KA, Conway DJ, Pinder M, Sirugo G, Sisay-Joof F, Usen S, Auburn S, Bumpstead SJ, Campino S, Coffey A, Dunham A, Fry AE, Green A, Gwilliam R, Hunt SE, Inouye M, Jeffreys AE, Mendy A, Palotie A, Potter S, Ragoussis J, Rogers J, Rowlands K, Somaskantharajah E, Whittaker P, Widden C, Donnelly P, Howie B, Marchini J, Morris A, SanJoaquin M, Achidi EA, Agbenyega T, Allen A, Amodu O, Corran P, Djimde A, Dolo A, Doumbo OK, Drakeley C, Dunstan S, Evans J, Farrar J, Fernando D, Hien TT, Horstmann RD, Ibrahim M, Karunaweera N, Kokwaro G, Koram KA, Lemnge M, Makani J, Marsh K, Michon P, Modiano D, Molyneux ME, Mueller I, Parker M, Peshu N, Plowe C V, Puijalon O, Reeder J, Reyburn H, Riley EM, Sakuntabhai A, Singhasivanon P, Sirima S, Tall A, Taylor TE, Thera M, Troye-Blomberg M, Williams TN, Wilson M, Kwiatkowski DP 2009. Genome-wide and fine-resolution association analysis of malaria in West Africa. *Nat Genet 41*: 657-665.

Jayavanth S, Singh M 2003. Artificial neural network analysis of malaria severity through aggregation and deformability parameters of erythrocytes. *Clin Hemorheol Microcirc 29*: 457-468.

Joos C, Marrama L, Polson HEJ, Corre S, Diatta A-M, Diouf B, Trape J-F, Tall A, Longacre S, Perraut R 2010. Clinical protection from falciparum malaria correlates with neutrophil respiratory bursts induced by merozoites opsonized with human serum antibodies. *PLoS One 5*: e9871.

Kapelski S, Klockenbring T, Fischer R, Barth S, Fendel R 2014. Assessment of the neutrophilic antibody-dependent respiratory burst (ADRB) response to *Plasmodium falciparum*. *J Leukoc Biol 96*: 1131-1142.

Karunaweera ND, Grau GE, Gamage P, Carter R, Mendis KN 1992. Dynamics of fever and serum levels of tumor necrosis factor are closely associated during clinical paroxysms in *Plasmodium vivax* malaria. *Proc Natl Acad Sci USA 89*: 3200-3203.

Kho S, Marfurt J, Noviyanti R, Kusuma A, Piera KA, Burdam FH, Kenangalem E, Lampah DA, Engwerda CR, Poespoprodjo JR, Price RN, Anstey NM, Minigo G, Woodberry T 2015. Preserved dendritic cell HLA-DR expression and reduced regulatory T cell activation in asymptomatic *Plasmodium falciparum* and *P. vivax* infection. *Infect Immun 83*: 3224-3232.

Kiang R, Adimi F, Soika V, Nigro J, Singhasivanon P, Sirichaisinthop J, Leemingsawat S, Apiwathnasorn C, Looareesuwan S 2006. Meteorological, environmental remote sensing and neural network analysis of the epidemiology of malaria transmission in Thailand. *Geospat Health 1*: 71-84.

Kinyanjui SM, Mwangi T, Bull PC, Newbold CI, Marsh K 2004. Protection against clinical malaria by heterologous immunoglobulin G antibodies against malaria-infected erythrocyte variant surface antigens requires interaction with asymptomatic infections. *J Infect Dis 190*: 1527-1533.

Koukouikila-Koussounda F, Ntoumi F, Ndounga M, Tong H V, Abena A-A, Velavan TP 2013. Genetic evidence of regulatory gene variants of the STAT6, IL10R and FOXP3 locus as a susceptibility factor in uncomplicated malaria and parasitaemia in Congolese children. *Malar J 12*: 9.

Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, Moestrup SK 2001. Identification of the haemoglobin scavenger receptor. *Nature 409*: 198-201.

Kublin JG, Patnaik P, Jere CS, Miller WC, Hoffman IF, Chimbiya N, Pendame R, Taylor TE, Molyneux ME 2005. Effect of *Plasmodium falciparum* malaria on concentration of HIV-1-RNA in the blood of adults in rural Malawi: a prospective cohort study. *Lancet 365*: 233-240.

Kuesap J, Hirayama K, Kikuchi M, Ruangweerayut R, Na-Bangchang K 2010. Study on association between genetic polymorphisms of haem oxygenase-1, tumour necrosis factor, cadmium exposure and malaria pathogenicity and severity. *Malar J 9*: 260.

Kwiatkowski D 2000. Genetic susceptibility to malaria getting complex. *Curr Opin Genet. Dev 10*: 320-324.

Kwiatkowski D, Hill A V, Sambou I, Twumasi P, Castracane J, Manogue KR, Cerami A, Brewster DR, Greenwood BM 1990. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet 336*: 1201-1204.

Ladeia-Andrade S, Ferreira MU, Carvalho ME de, Curado I, Coura JR 2009. Age-dependent acquisition of protective immunity to malaria in riverine populations of the Amazon Basin of Brazil. *Am J Trop Med Hyg 80*: 452-459.

Laishram DD, Sutton PL, Nanda N, Sharma VL, Sobti RC, Carlton JM, Joshi H 2012. The complexities of malaria disease manifestations with a focus on asymptomatic malaria. *Malar J 11*: 29.

Lemaitre M, Watier L, Briand V, Garcia A, Hesran JY Le, Cot M 2014. Coinfection with *Plasmodium falciparum* and *Schistosoma haematobium*: additional evidence of the protective effect of schistosomiasis on malaria in Senegalese children. *Am J Trop Med Hyg 90*: 329-334.

Lindblade KA, Steinhardt L, Samuels A, Kachur SP, Slutsker L 2013. The silent threat: asymptomatic parasitemia and malaria transmission. *Expert Rev Anti Infect Ther 11*: 623-639.

Martin SK, Miller LH, Hicks CU, David-West A, Ugbode C, Deane M 1979. Frequency of blood group antigens in Nigerian children with falciparum malaria. *Trans R Soc Trop. Med Hyg 73*: 216-218.

Medzhitov R, Schneider DS, Soares MP 2012. Disease tolerance as a defense strategy. *Science 335*: 936-941.

Mendonça VR, Souza LC, Garcia GC, Magalhães BM, Lacerda MV, Andrade BB, Gonçalves MS, Barral-Netto M 2014. DDX39B (BAT1), TNF and IL6 gene polymorphisms and association with clinical outcomes of patients with *Plasmodium vivax* malaria. *Malar J 13*: 278.

Mendonça VRR de, Goncalves MS, Barral-Netto M 2012a. The host genetic diversity in malaria infection. *J Trop Med 2012*: 940616.

Mendonça VR R, Luz NF, Santos NJG, Borges VM, Gonçalves MS, Andrade BB, Barral-Netto M 2012b. Association between the haptoglobin and heme oxygenase 1 genetic profiles and soluble CD163 in susceptibility to and severity of human malaria. *Infect Immun 80*: 1445-1454.

Mendonça VRR, Queiroz ATL, Lopes FM, Andrade BB, Barral-Netto M 2013. Networking the host immune response in *Plasmodium vivax* malaria. *Malar J 12*: 69.

Milet J, Nuel G, Watier L, Courtin D, Slaoui Y, Senghor P, Migot-Nabias F, Gaye O, Garcia A 2010. Genome wide linkage study, using a 250K SNP map, of *Plasmodium falciparum* infection and mild malaria attack in a Senegalese population. *PLoS One 5*: e11616.

Miller YI, Shaklai N 1999. Kinetics of hemin distribution in plasma reveals its role in lipoprotein oxidation. *Biochim Biophys Acta 1454*: 153-164.

Minigo G, Woodberry T, Piera KA, Salwati E, Tjitra E, Kenangalem E, Price RN, Engwerda CR, Anstey NM, Plebanski M 2009. Parasite-dependent expansion of TNF receptor II-positive regulatory T-cells with enhanced suppressive activity in adults with severe malaria. *PLoS Pathog 5*: e1000402.

Mombo L-E, Ntoumi F, Bisseye C, Ossari S, Lu CY, Nagel RL, Krishnamoorthy R 2003. Human genetic polymorphisms and asymptomatic *Plasmodium falciparum* malaria in Gabonese schoolchildren. *Am J Trop Med Hyg 68*: 186-190.

Moormann AM, Sumba PO, Chelimo K, Fang H, Tisch DJ, Dent AE, John CC, Long CA, Vulule J, Kazura JW 2013. Humoral and cellular immunity to *Plasmodium falciparum* merozoite surface protein 1 and protection from infection with blood-stage parasites. *J Infect Dis 208*: 149-158.

Mordmüller BG, Metzger WG, Juillard P, Brinkman BM, Verweij CL, Grau GE, Kremsner PG 1997. Tumor necrosis factor in *Plasmodium falciparum* malaria: high plasma level is associated with fever, but high production capacity is associated with rapid fever clearance. *Eur Cytokine Netw 8*: 29-35.

Najem GR, Sulzer AJ 2003. Transfusion-induced malaria from an asymptomatic carrier. *Transfusion 16*: 473-476.

Ntoumi F, Contamin H, Rogier C, Bonnefoy S, Trape JF, Mercereau-Puijalon O 1995. Age-dependent carriage of multiple *Plasmodium falciparum* merozoite surface antigen-2 alleles in asymptomatic malaria infections. *Am J Trop Med Hyg 52*: 81-88.

Nussenzweig RS, Vanderberg J, Most H, Orton C 1967. Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei*. *Nature 216*: 160-162.

Olotu A, Moris P, Mwacharo J, Vekemans J, Kimani D, Janssens M, Kai O, Jongert E, Lievens M, Leach A, Villafana T, Savarese B, Marsh K, Cohen J, Bejon P 2011. Circumsporozoite-specific T-cell responses in children vaccinated with RTS,S/AS01E and protection against *P. falciparum* clinical malaria. *PLoS One 6*: e25786.

Ong'echa JM, Davenport GC, Vulule JM, Hittner JB, Perkins DJ 2011. Identification of inflammatory biomarkers for pediatric malarial anemia severity using novel statistical methods. *Infect Immun 79*: 4674-4680.

Oppenheimer SJ, Higgs DR, Weatherall DJ, Barker J, Spark RA 1984. Alpha thalassaemia in Papua New Guinea. *Lancet 1*: 424-426.

Overstreet MG, Cockburn IA, Chen Y-C, Zavala F 2008. Protective CD8 T-cells against *Plasmodium* liver stages: immunobiology of an "unnatural" immune response. *Immunol Rev 225*: 272-283.

Palm NW, Medzhitov R 2009. Pattern recognition receptors and control of adaptive immunity. *Immunol Rev 227*: 221-233.

Pamplona A, Ferreira A, Balla J, Jeney V, Balla G, Epiphanio S, Chora A, Rodrigues CD, Gregoire IP, Cunha-Rodrigues M, Portugal S, Soares MP, Mota MM 2007. Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nat Med 13*: 703-710.

Paoli M, Anderson BF, Baker HM, Morgan WT, Smith A, Baker EN 1999. Crystal structure of hemopexin reveals a novel high-affinity heme site formed between two beta-propeller domains. *Nat Struct Biol 6*: 926-931.

Perdijk O, Arama C, Giusti P, Maiga B, Troye-Blomberg M, Dolo A, Doumbo O, Persson J-O, Boström S 2013. Haptoglobin phenotype prevalence and cytokine profiles during *Plasmodium falciparum* infection in Dogon and Fulani ethnic groups living in Mali. *Malar J 12*: 432.

Perkins DJ, Hittner JB, Mwaikambo ED, Granger DL, Weinberg JB, Anstey NM 2005. Impaired systemic production of prostaglandin E2 in children with cerebral malaria. *J Infect Dis 191*: 1548-1557.

Philippidis P, Mason JC, Evans BJ, Nadra I, Taylor KM, Haskard DO, Landis RC 2004. Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxygenase-1 synthesis: antiinflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. *Circ Res 94*: 119-126.

Portugal S, Doumtabe D, Traore B, Miller LH, Troye-Blomberg M, Doumbo OK, Dolo A, Pierce SK, Crompton PD 2012. B-cell analysis of ethnic groups in Mali with differential susceptibility to malaria. *Malar J 11*: 162.

Portugal S, Tipton CM, Sohn H, Kone Y, Wang J, Li S, Skinner J, Virtaneva K, Sturdevant DE, Porcella SF, Doumbo OK, Doumbo S, Kayentao K, Ongoiba A, Traore B, Sanz I, Pierce SK, Crompton PD 2015. Malaria-associated atypical memory B-cells exhibit markedly reduced B-cell receptor signaling and effector function. *Elife* doi: 10.7554/eLife.07218.

Powrie F, Read S, Mottet C, Uhlig H, Maloy K 2003. Control of immune pathology by regulatory T-cells. *Novartis Found Symp 252*: 92-98; discussion 98-105, 106-114.

Quaye IK 2008. Haptoglobin, inflammation and disease. *Trans R Soc Trop Med Hyg 102*: 735-742.

Rihet P, Traoré Y, Abel L, Aucan C, Traoré-Leroux T, Fumoux F 1998. Malaria in humans: *Plasmodium falciparum* blood infection levels are linked to chromosome 5q31-q33. *Am J Hum Genet 63*: 498-505.

Rodrigues M, Nussenzweig RS, Zavala F 1993. The relative contribution of antibodies, $CD4^+$ and $CD8^+$ T-cells to sporozoite-induced protection against malaria. *Immunology 80*: 1-5.

Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJF, van Gemert GJ, van de Vegte-Bolmer M, van Schaijk B, Teelen K, Arens T, Spaarman L, Mast Q de, Roeffen W, Snounou G, Rénia L, van der Ven A, Hermsen CC, Sauerwein R 2009. Protection against a malaria challenge by sporozoite inoculation. *N Engl J Med 361*: 468-477.

Roestenberg M, Teirlinck AC, McCall MBB, Teelen K, Makamdop KN, Wiersma J, Arens T, Beckers P, van Gemert G, van der Vegte-Bolmer M, van der Ven AJAM, Luty AJF, Hermsen CC, Sauerwein RW 2011. Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. *Lancet 377*: 1770-1776.

Romero P, Maryanski JL, Corradin G, Nussenzweig RS, Nussenzweig V, Zavala F 1989. Cloned cytotoxic T-cells recognize an epitope in the circumsporozoite protein and protect against malaria. *Nature 341*: 323-326.

Rono J, Osier FHA, Olsson D, Montgomery S, Mhoja L, Rooth I, Marsh K, Färnert A 2013. Breadth of anti-merozoite antibody responses is associated with the genetic diversity of asymptomatic *Plasmodium falciparum* infections and protection against clinical malaria. *Clin Infect Dis 57*: 1409-1416.

Sabchareon A, Burnouf T, Ouattara D, Attanath P, Bouharoun-Tayoun H, Chantavanich P, Foucault C, Chongsuphajaisiddhi T, Druilhe P 1991. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. *Am J Trop Med Hyg 45*: 297-308.

Sagna AB, Gaayeb L, Sarr JB, Senghor S, Poinsignon A, Boutouaba-Combe S, Schacht A-M, Hermann E, Faye N, Remoue F, Riveau G 2013. *Plasmodium falciparum* infection during dry season: IgG responses to *Anopheles gambiae* salivary gSG6-P1 peptide as sensitive biomarker for malaria risk in Northern Senegal. *Malar J 12*: 301.

Schmidt NW, Podyminogin RL, Butler NS, Badovinac VP, Tucker BJ, Bahjat KS, Lauer P, Reyes-Sandoval A, Hutchings CL, Moore AC, Gilbert SC, Hill A V, Bartholomay LC, Harty JT 2008. Memory CD8 T-cell responses exceeding a large but definable threshold provide long-term immunity to malaria. *Proc Natl Acad Sci USA 105*: 14017-14022.

Schneider P, Bousema JT, Gouagna LC, Otieno S, Vegte-Bolmer M van de, Omar SA, Sauerwein RW 2007. Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *Am J Trop Med Hyg 76*: 470-474.

Schofield L, Villaquiran J, Ferreira A, Schellekens H, Nussenzweig R, Nussenzweig V 1987. Gamma interferon, $CD8^+$ T-cells and antibodies required for immunity to malaria sporozoites. *Nature 330*: 664-666.

Scuracchio P, Vieira SD, Dourado DA, Bueno LM, Colella R, Ramos-Sanchez EM, Lima GFMC, Inoue J, Sanchez MCA, Santi SM Di 2011. Transfusion-transmitted malaria: case report of asymptomatic donor harboring *Plasmodium malariae*. *Rev Inst Med Trop Sao Paulo 53*: 55-59.

Shimizu H, Tamam M, Soemantri A, Ishida T 2005. Glucose-6-phosphate dehydrogenase deficiency and Southeast Asian ovalocytosis in asymptomatic *Plasmodium* carriers in Sumba island, Indonesia. *J Hum Genet 50*: 420-424.

Sikora M, Laayouni H, Menendez C, Mayor A, Bardaji A, Sigauque B, Netea MG, Casals F, Bertranpetit J 2011. A targeted association study of immunity genes and networks suggests novel associations with placental malaria infection. *PLoS One 6*: e24996.

Smith T, Felger I, Tanner M, Beck HP 1999. Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections. *Trans R Soc Trop Med Hyg 93* (Suppl. 1): 59-64.

Stephens R, Albano FR, Quin S, Pascal BJ, Harrison V, Stockinger B, Kioussis D, Weltzien H-U, Langhorne J 2005. Malaria-specific transgenic CD4(+) T-cells protect immunodeficient mice from lethal infection and demonstrate requirement for a protective threshold of antibody production for parasite clearance. *Blood 106*: 1676-1684.

Stephens R, Langhorne J 2010. Effector memory Th1 CD4 T-cells are maintained in a mouse model of chronic malaria. *PLoS Pathog 6*: e1001208.

Tada MS, Ferreira RGM, Katsuragawa TH, Martha RCD, Costa JDAN, Albrecht L, Wunderlich G, da Silva LHP 2012. Asymptomatic infection with *Plasmodium falciparum* and *Plasmodium vivax* in the Brazilian Amazon Basin: to treat or not to treat? *Mem Inst Oswaldo Cruz 107*: 621-629.

Taylor SM, Cerami C, Fairhurst RM 2013. Hemoglobinopathies: slicing the Gordian knot of *Plasmodium falciparum* malaria pathogenesis. *PLoS Pathog 9*: e1003327.

Thornton AM, Shevach EM 2000. Suppressor effector function of $CD4^+CD25^+$ immunoregulatory T-cells is antigen nonspecific. *J Immunol 164*: 183-190.

Timmann C, Evans JA, König IR, Kleensang A, Rüschendorf F, Lenzen J, Sievertsen J, Becker C, Enuameh Y, Kwakye KO, Opoku E, Browne ENL, Ziegler A, Nürnberg P, Horstmann RD 2007. Genome-wide linkage analysis of malaria infection intensity and mild disease. *PLoS Genet 3*: e48.

Timmann C, Thye T, Vens M, Evans J, May J, Ehmen C, Sievertsen J, Muntau B, Ruge G, Loag W, Ansong D, Antwi S, Asafo-Adjei E, Nguah SB, Kwakye KO, Akoto AOY, Sylverken J, Brendel M, Schuldt K, Loley C, Franke A, Meyer CG, Agbenyega T, Ziegler A, Horstmann RD 2012. Genome-wide association study indicates two novel resistance *loci* for severe malaria. *Nature 489*: 443-446.

Tolosano E, Fagoonee S, Morello N, Vinchi F, Fiorito V 2010. Heme scavenging and the other facets of hemopexin. *Antioxid Redox Signal 12*: 305-320.

Tran TM, Li S, Doumbo S, Doumtabe D, Huang C-Y, Dia S, Bathily A, Sangala J, Kone Y, Traore A, Niangaly M, Dara C, Kayentao K, Ongoiba A, Doumbo OK, Traore B, Crompton PD 2013. An intensive longitudinal cohort study of Malian children and adults reveals no evidence of acquired immunity to *Plasmodium falciparum* infection. *Clin Infect Dis 57*: 40-47.

Tran TM, Samal B, Kirkness E, Crompton PD 2012. Systems immunology of human malaria. *Trends Parasitol 28*: 248-257.

Trape JF, Rogier C, Konate L, Diagne N, Bouganali H, Canque B, Legros F, Badji A, Ndiaye G, Ndiaye P 1994. The Dielmo Project: a longitudinal study of natural malaria infection and the mechanisms of protective immunity in a community living in a holoendemic area of Senegal. *Am J Trop Med Hyg 51*: 123-137.

Tsuji M, Bergmann CC, Takita-Sonoda Y, Murata K, Rodrigues EG, Nussenzweig RS, Zavala F 1998. Recombinant Sindbis viruses expressing a cytotoxic T-lymphocyte epitope of a malaria parasite or of influenza virus elicit protection against the corresponding pathogen in mice. *J Virol 72*: 6907-6910.

Verhoeff FH, Brabin BJ, Hart CA, Chimsuku L, Kazembe P, Broadhead RL 1999. Increased prevalence of malaria in HIV-infected pregnant women and its implications for malaria control. *Trop Med Int Health 4*: 5-12.

Villasis E, Lopez-Perez M, Torres K, Gamboa D, Neyra V, Bendezu J, Tricoche N, Lobo C, Vinetz JM, Lustigman S 2012. Anti-*Plasmodium falciparum* invasion ligand antibodies in a low malaria transmission region, Loreto, Peru. *Malar J 11*: 361.

Walther M, Caul A De, Aka P, Njie M, Amambua-Ngwa A, Walther B, Predazzi IM, Cunnington A, Deininger S, Takem EN, Ebonyi A, Weis S, Walton R, Rowland-Jones S, Sirugo G, Williams SM, Conway DJ 2012. HMOX1 gene promoter alleles and high HO-1 levels are associated with severe malaria in Gambian children. *PLoS Pathog 8*: e1002579.

Walther M, Tongren JE, Andrews L, Korbel D, King E, Fletcher H, Andersen RF, Bejon P, Thompson F, Dunachie SJ, Edele F, de Souza JB, Sinden RE, Gilbert SC, Riley EM, Hill AVS 2005. Upregulation of TGF-beta, FOXP3, and $CD4^+CD25^+$ regulatory T-cells correlates with more rapid parasite growth in human malaria infection. *Immunity 23*: 287-296.

Wammes LJ, Wiria AE, Toenhake CG, Hamid F, Liu KY, Suryani H, Kaisar MMM, Verweij JJ, Sartono E, Supali T, Smits HH, Luty AJ, Yazdanbakhsh M 2013. Asymptomatic plasmodial infection is associated with increased tumor necrosis factor receptor II-expressing regulatory T-cells and suppressed type 2 immune responses. *J Infect Dis* 207: 1590-1599.

Weiss GE, Crompton PD, Li S, Walsh LA, Moir S, Traore B, Kayentao K, Ongoiba A, Doumbo OK, Pierce SK 2009. Atypical memory B-cells are greatly expanded in individuals living in a malaria-endemic area. *J Immunol 183*: 2176-2182.

White NJ 2008. The role of anti-malarial drugs in eliminating malaria. *Malar J 7* (Suppl.I): S8.

Whitworth J, Morgan D, Quigley M, Smith A, Mayanja B, Eotu H, Omoding N, Okongo M, Malamba S, Ojwiya A 2000. Effect of HIV-1 and increasing immunosuppression on malaria parasitaemia and clinical episodes in adults in rural Uganda: a cohort study. *Lancet 356*: 1051-1056.

WHO - World Health Organization 2014. World Malaria Report 2014. Available from: who.int/malaria/publications/world_malaria_report_2014/en/.

Wykes MN, Liu XQ, Beattie L, Stanisic DI, Stacey KJ, Smyth MJ, Thomas R, Good MF 2007. *Plasmodium* strain determines dendritic cell function essential for survival from malaria. *PLoS Pathog 3*: e96.

6. DISCUSSÃO

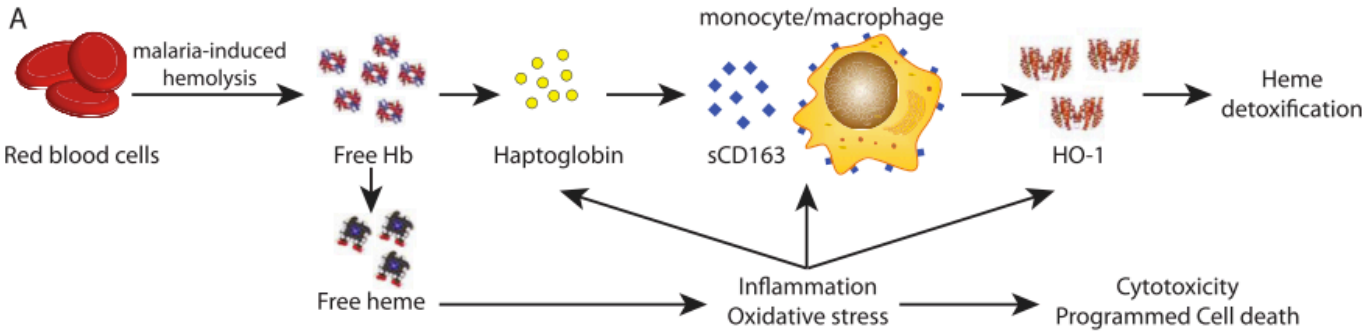
Esta Tese apresenta uma coletânea de sete artigos que auxiliam na compreensão dos determinantes imunológicos relacionados à gravidade e resistência à malária pelo *P. vivax,* e nos casos coinfectados com dengue, na Região Amazônica Brasileira.

Alterações genéticas e nos níveis plasmáticos de moléculas envolvidas na via de metabolização do heme podem explicar a patogênese associada a malária. Diversos estudos, sobretudo em modelos experimentais, têm associado moléculas na via de detoxificação do heme com as manifestações da malária (FERREIRA et al., 2008; GOZZELINO; JENEY; SOARES, 2010; SEIXAS et al., 2009). Nossos resultados indicaram que o fenótipo Hp2.2 da haptoglobina está associado ao risco maior de desenvolver malária sintomática quando comparada com casos assintomáticos. O Hp2.2 tem sido associado a maior susceptibilidade a condições inflamatórias diversas, incluindo a malária (ATKINSON et al., 2006; CHEN et al., 2011b; COX et al., 2007; FRIIS et al., 2003; KASVOSVE et al., 2000). A presença do genotipo Hp2.2 está relacionada com o aumento no estresse oxidativo comparado com o Hp1.1 (ASLEH et al., 2005; MELAMED-FRANK et al., 2001). Além disso, o complexo Hb-Hp2.2, e não os complexos com o alelo *Hp1*, podem ser internalizados por monócitos e estimular a produção de citocinas pró-inflamatórias (ROGERSON, 2006). A haptoglobina é considerada uma proteína de fase aguda e está aumenta em resposta à inflamação (IMRIE et al., 2007). Desta maneira, nós observamos que os níveis de heme e Hp estão aumentados em indíviduos com malária

sintomática quando comparados com os casos de malária assintomáticos ou grupo controle. O complexo Hp-Hb é reconhecido via CD163 expresso em macrófagos e monócitos; este receptor pode também ser encontrado na forma solúvel em condições inflamatórias. Nossos resultados demonstraram que níveis plasmáticos de sCD163 aumentam gradualmente com a gravidade da malária. O sCD163 tem sido identificado como um mediador anti-inflamatório que inibe a ativação e proliferação de linfócitos T, e a ligação de complexos Hp-Hp com o sCD163 parece diminuir o suprimento de ferro (presente no heme) disponível para bactérias hemolíticas (HÖGGER et al., 1998; WEAVER et al., 2006). Desta forma, os níveis aumentados de sCD163 encontrados nos indivíduos sintomáticos no nosso trabalho pode ser um mecanismo regulatório contra a inflamação.

A HO-1, enzima responsável por detoxificar o organismo do heme livre, apresenta um polimorfismo microssatélite que pode alterar a sua expressão. Nós identificamos que indíviduos com o componente longo (>30 repetições GT) do polimorfismo da *HMOX1* tiveram susceptibilidade maior para desenvolver malária sintomática quando comparados com aqueles com o componente curto (<30 repetições GT). Em contraste, outros estudos encontraram repetições GT curtas em pacientes com malária cerebral comparados com grupos de malária não-complicada ou controle (SAMBO et al., 2010; TAKEDA et al., 2005). Vale ressaltar que os pacientes com malária do nosso estudo são infectados pelo *P. vivax* e apresentam mais frequentemente formas amenas da doença. No que se refere aos níveis plasmáticos da HO-1, nós encontramos que, em geral, os indivíduos com malária sintomática têm concentrações maiores que os demais grupos.

Níveis elevados da HO-1 também já foram descritos em outras doenças com caráter inflamatório (BAO et al., 2012; BLANN et al., 2011; CHEN et al., 2011a; KIRINO et al., 2005; MIYAZAKI et al., 2010). Alguns estudos em modelos experimentais sugerem que a deleção do gene *HMOX1* ou a inibição farmacológica da HO-1 estão relacionados com a patogênese da malária, visto que os camundongos não teriam a enzima responsável por detoxificar o heme livre deletério (FERREIRA et al., 2008). Entretanto, os níveis séricos elevados da HO-1 encontrados nos indivíduos com malária sintomática do nosso estudo pode ser um efeito regulatório induzido pelo heme, especialmente considerando que a expressão da *HMOX1* é altamente induzida pelo heme (TENHUNEN; MARVER; SCHMID, 1970). Um resumo das alterações encontradas na via do heme em nosso estudo pode ser visualizada na Figura 1 abaixo (publicada em nosso manuscrito).



B

	Free heme levels	Haptoglobin levels	*Hp* genetic profile	sCD163 levels	HO-1 levels	*HMOX1* GT repeats	Inflammatory scores
Asymptomatic malaria	↑	↑	*Hp1S* allele	unchanged	unchanged	short	low
Symptomatic malaria	↑↑	↑↑	*Hp2* allele and *Hp2.2* genotype	↑↑	↓ or ↑↑	long	high

Figura 1. Metabolismo do heme e os desfechos clínicos da malária. (Fonte: Mendonça et al. 2012)

A resposta imune dos indivíduos infectados pelo *P. vivax* ainda permanece pouco conhecida, e, recentemente, a análise por redes tem permitido um melhor entendimento das interações entre os componentes do sistema imune. Nós utilizamos este tipo de análise para verificar as correlações entre biomarcadores plasmáticos (citocinas, quimiocinas, marcadores de disfunção orgânica e outras proteínas de resposta inflamatória) nas diferentes manifestações clínicas da malária vivax, incluindo grupos de individuos não-infectados, malária não-complicada, malária assintomática, malária grave, e malária grave que evoluiu para o óbito (Figura 2, publicada em nosso manuscrito). No nosso estudo, os indivíduos com malária assintomática tiveram a maior densidade de interações das redes em relação aos demais grupos, o que sugere existir um mecanismo regulatório complexo por trás da susceptibilidade e resistência à malária. Os valores de densidade das redes dos nossos dados são similares às outras redes entre proteínas e vias de metabolismo descritas em diversos contextos clínicos (FRANKENSTEIN; ALON; COHEN, 2006).

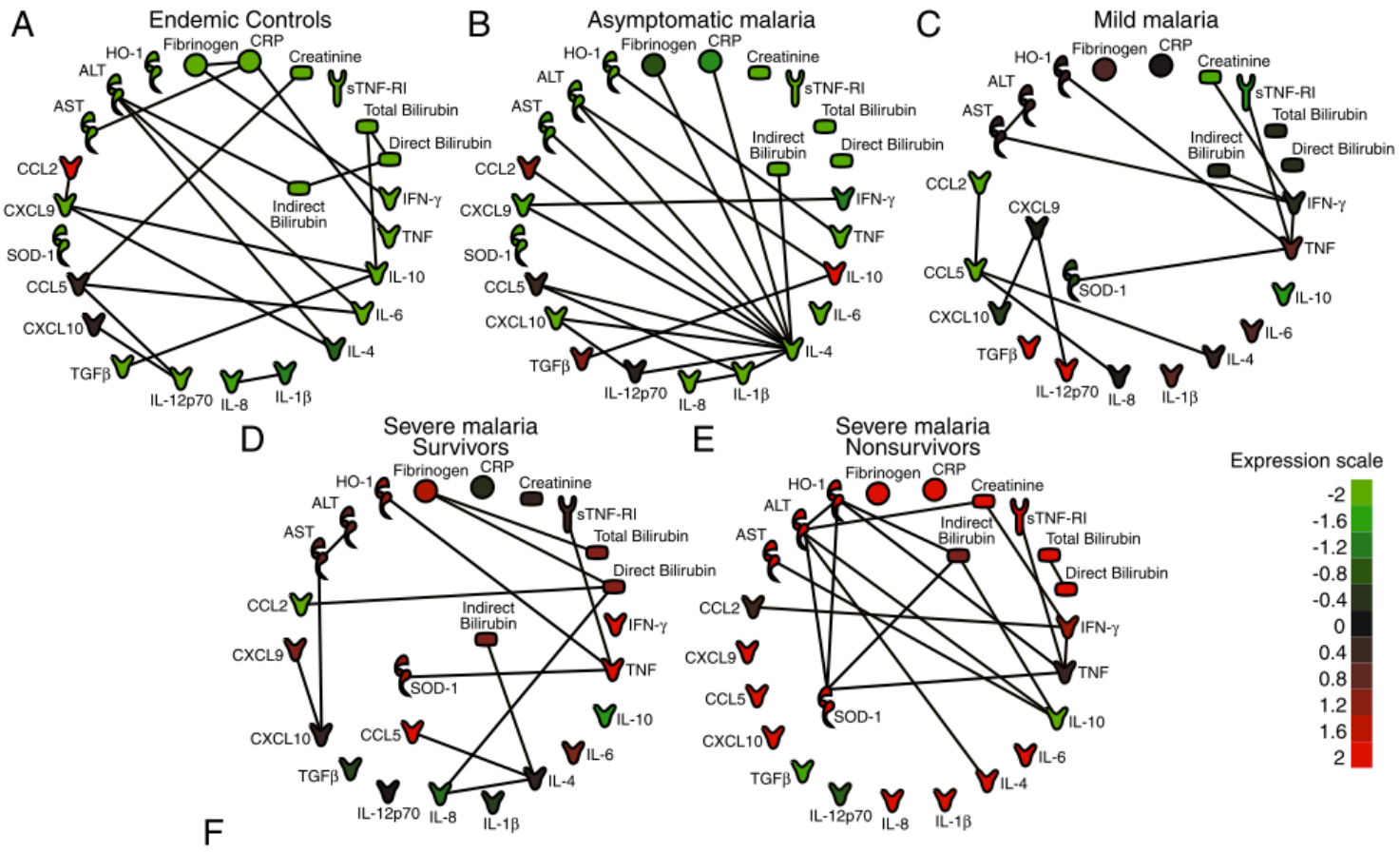


Figura 2. Redes dos biomarcadores inflamatórios na malária por *P. vivax*. (Adaptado de Mendonça et al. 2013).

A alta conectividade exibida pelo CXCL9 em nossos resultados pode ser indicativo de um papel importante desta quimiocina no grupo de indivíduos não-infectados, visto que esta molécula foi correlacionada com proteção contra a malária em voluntários imunizados pela vacina contra a proteína circunsporozoíta do plasmódio (DUNACHIE et al., 2010). IL-10 e IL-4 tiveram interações significativas com o CXCL9, que também interagiu com o motivo C-C ligante (CCL)2 e IFN-γ no grupo não-infectado e assintomático, respectivamente, evidenciando que estas citocinas podem modular o efeito de mediadores pró-inflamatórios. Um estudo *in vitro* com macrófagos murinos demonstrou que o IL-4 suprime a expressão de CXCL9 e outros genes

induzidos pelo IFN-γ através da sinalização pelo STAT-6 (OHMORI; HAMILTON, 1998). De modo intrigante, o grupo de malária assintomática apresentou um papel importante para a IL-4, com diversas interações envolvendo esta citocina. O potencial papel anti-inflamatório da IL-4 na resposta imune durante a malária não é completamente compreendido (CABANTOUS et al., 2009). A IL-4 parece auxiliar na imunidade anti-esporozoíta e estudos em camundondos infectados pelo *P. yoelli* sugerem que esta citocina é necessária para a produção de linfócitos T CD8+ (CARVALHO et al., 2002) e para o desenvolvimento de memória contra o estágio hepático do plasmódio (MORROT et al., 2005). A número elevado de interações entre o IL-4 e outros biomarcadores em portadores assintomáticos de malária sugere uma possível papel desta molécula em mediar a tolerância de indivíduos constantemente expostos ao *P. vivax*.

No grupo de malária não-complicada do nosso estudo, IFN-γ, TNF e CCL5 demonstraram ser biomarcadores importantes no perfil da rede destes indivíduos. A CCL5 é uma quimiocina envolvida na geração de infiltrados celulares inflamatórios, e níveis baixos desta quimiocina estão associados com malária cerebral e anemia grave pela malária (JOHN et al., 2006; WERE et al., 2006, 2009). A SOD-1, que já foi previamente associada à malária pelo *P. vivax* (ANDRADE et al., 2010b), apresentou conexões apenas nos grupos de doença sintomática, especialmente nos indivíduos que evoluíram para o óbito. O acúmulo de SOD-1 sem o aumento compensatório das enzimas catalase ou glutationa redutase podem levar ao acúmulo de água oxigenada e a produção de mais radicais livres (MARIKOVSKY et al., 2003). Além disso, a liberação de heme durante o ciclo eritrocítico da malária diminui a produção

das moléculas anti-inflamatórias PGE2 e TGF-β de células mononucleares através da SOD-1 (ANDRADE et al., 2010d). Desta maneira, a conectividade da SOD-1 observada nos pacientes graves pode ser uma resposta em relação à produção de heme livre deletério durante a hemólise intravascular. Adicionalmente, nos pacientes com malária grave do nosso estudo, a parasitemia apresentou diversas interações positivas com mediadores inflamatórios previamente descritos em participar da imunopatogênese da malária, como o TNF, IFN-γ, IL-10 e TGF-β (ANDRADE; BARRAL-NETTO, 2011; ANDRADE et al., 2010a, 2010b). Surpreendentemente, transaminases hepáticas, bilirrubinas, creatinina e proteína C reativa não foram correlacionados com a parasitemia. A falta de interações diretas entre a parasitemia e os marcadores laboratoriais de inflamação tecidual e estresse oxidativo sugere que a carga parasitária pode influenciar mais a resposta imune do hospedeiro do que induzir diretamente dano tecidual.

A malária exerce uma significativa pressão seletiva no genoma humano. Esta doença é a força evolucionária por trás de doenças, como a doença falciforme, talassemias e deficiência de glicose-6-fosfato (KWIATKOWSKI, 2005). Em estudo de revisão, nós encontramos que polimorfimos nos eritrócitos, mediadores do sistema imune, e moleculas envolvidas em mecanismos de citoaderência influenciam o desfecho clínico da malária (FIgura 3, publicada em nosso manuscrito). O principal objetivo da epidemiologia genética é identificar as vias moleculares críticas na patogênese e imunidade, possibilitando a geração de novas estratégias para tratamento e/ou prevenção de doenças (KWIATKOWSKI, 2000). Sendo assim, nós investigamos se alterações genéticas em genes relacionados ao

sistema imune poderiam influenciar os desfechos clínicos da malária vivax. Polimorfismos no *DDX39B* (*BAT1*) têm sido descritos em diversas doenças com perfis inflamatórios, incluindo neuropatia, miastenia gravis, alergias, doença de Alzheimer, infarto do miocárdio, hepatites, artrite reumatóide, doença de Chagas, entre outras (CHERRY et al., 2008; DEGLI-ESPOSTI; LEELAYUWAT; DAWKINS, 1992; GNJEC et al., 2008; KOCH et al., 2007; OLIVEIRA et al., 2011; QUIÑONES-LOMBRAÑA et al., 2008; RAMASAWMY et al., 2006). No nosso estudo, encontrou-se que o alelo G do *DDX39B*-22C>G pode ser um fator de resistência à malária e o alelo C um fator de risco para complicações da doença. Polimorfismos na regiao promotora do *DDX39B* alteram a ligação de fatores transcricionais (YY1 e Oct1) e podem afetar a transcrição deste gene, sendo as sequências com os alelos -22G e -348T expressas mais eficientemente que as sequências contendo os alelos -22C e -348C (PRICE et al., 2004). Um estudo *in vitro* descobriu que o BAT1 parece diminuir a expressão de TNF e IL-6 (ALLCOCK; WILLIAMS; PRICE, 2001); deste modo, o alelo G do *DDX39B*-22C>G, que aumenta a expressão do BAT1, pode ser protetor contra a malária complicada por diminuir a expressão de citocinas pró-inflamatórias.

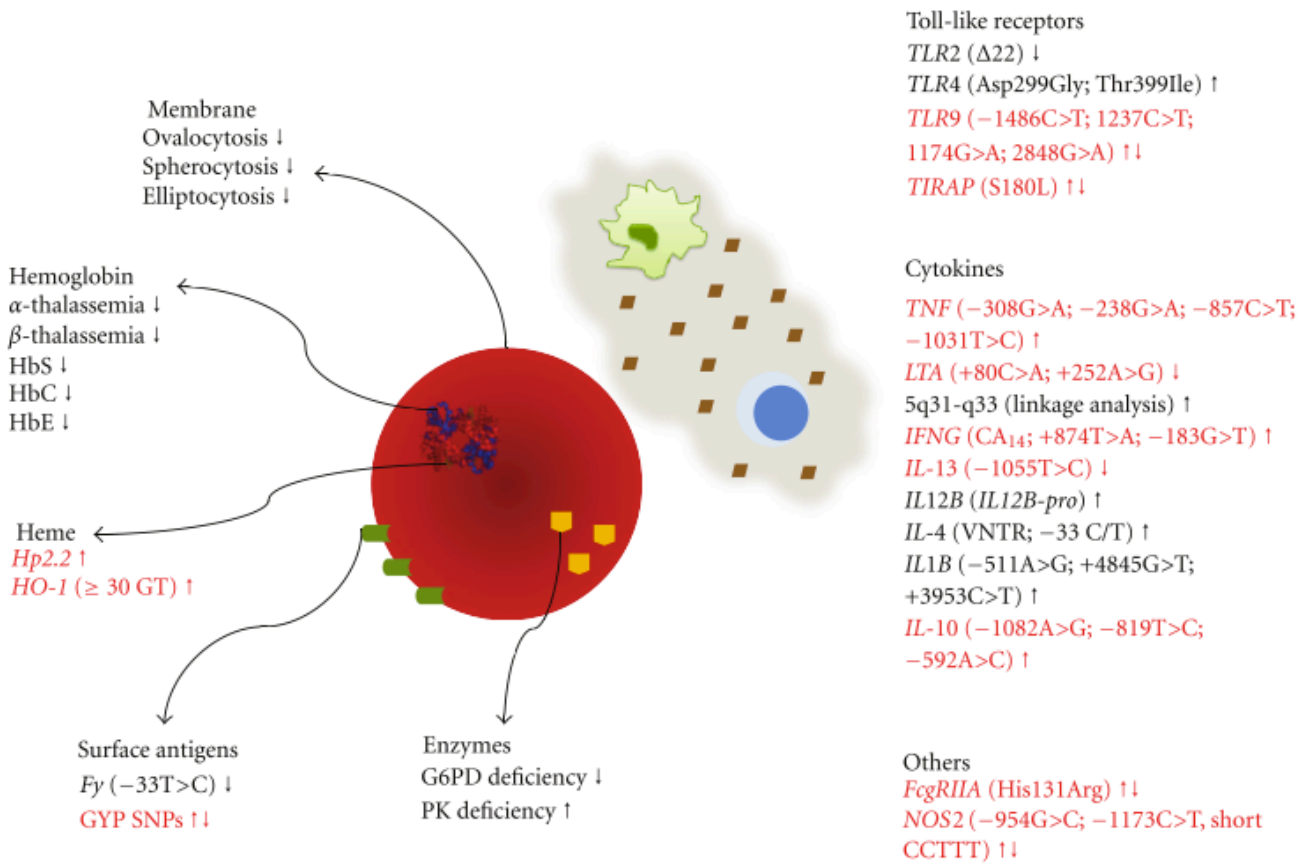


Figura 3. Influência de alterações genéticas nos eritrócitos e na resposta imune na malária. Setas para cima indicam susceptibilidade, setas para baixo, resistência. Resultados contraditórios ou não confimados destacados em vermelho. (Fonte: Mendonça et al. 2012).

Embora os SNPs *IL6*-176G>C e *TNF*-308G>A, descritos em nosso estudo, não estarem associados diretamente às manifestações clínicas da malária, estes podem indiretamente influenciar a doença por alterar os níveis de mediadores inflamatórios envolvidos na sua imunopatologia. Níveis elevados de TNF estão relacionados a patogênese de sintomas associados à malária, como a febre, e com as formas graves da infecção, como a malária cerebral e anemia grave (KARUNAWEERA et al., 1992). Nós encontramos que os participantes heterozigotos (AG) para *TNF*-308G>A tinham concentração plasmática de TNF maior que os participantes homozigotos

selvagens (GG). O alelo A desse SNP tem sido associado a um aumento na produção de TNF em diversos estudos (BOUMA et al., 1996; CANDORE et al., 1994; JACOB et al., 1990; WILSON et al., 1993). Uma única mutação frequentemente não é suficiente para predizer susceptibilidade ou resistência à malária; uma alternativa é a análise de haplótipos dos alelos mutantes no mesmo cromossoma. *DDX39B* é situado na região central do complexo principal de histocompatibilidade no braço curto do cromossoma humano 6, a uma distância aproximada de 150kb do gene do *TNF*. Apesar do nosso estudo não ter identificado nenhum haplótipo (envolvendo os polimorfismos do *DDX39B* [22C>G e 348C>T] e TNF-308G>A) que influncie o risco de malária, diversos haplótipos demonstraram aumentar a expressão de TNF, contribuindo indiretamente para a susceptibilidade à doença. De modo similar, encontramos que combinações de genótipos CG/CC/GG/GG e GG/CT/GG/GG, correspondendo aos respectivos polimorfismos *DDX39B*-22/ *DDX39B*-348/*TNF*-308/*IL6*-176, estão associados com risco diminuído e aumentado, respectivamente, de desenvolver manifestações clínicas durante a infecção pelo *P. vivax*. Além disso, os participantes do nosso estudo com a combinação de genótipos relacionada com a resistência (CG/CC/GG/GG) também apresentaram níveis diminuídos das citocinas pró-inflamatórias TNF e IL-6, sugerindo que o *DDX39B* confere proteção contra a patogênese da malária através da redução da resposta inflamatória.

Os três primeiros estudos desta Tese avaliaram a influência das interações entre citocinas pró e anti-inflamatórias, alterações genéticas em genes relacionados a resposta imune, assim como níveis séricos e polimorfismos de moléculas envolvidas na via de metabolização do heme,

com os diferentes desfechos clínicos da malária por *P. vivax*. Contudo, os mecanismos imunopatológicos do grupo de malária com hiperbilirrubinemia isolada permanecia desconhecido; sendo assim, nós tentamos desvendar a resposta imune desses indivíduos no próximo estudo. A hiperbilirrubinemia não foi mais considerada um critério de malária grave na última revisão feita pela OMS em 2010 (WHO, 2010), sendo considerada em diversos estudos apenas como uma condição associada a outros sinais/sintomas definidores de gravidade (ANAND; PURI, 2005; MISHRA et al., 2004; TRIPATHY et al., 2007). Adicionalmente, o dano hepático associado a malária com hiperbilirrubinemia pode alterar a expressão da hepcidina, hormônio responsável pela regulação do metabolismo do ferro (ANAND; PURI, 2005).

Em nosso estudo, indivíduos infectados pelo *P. vivax* com hiperbilirrubinemia tiveram níveis de IL-2 e IL-13 menores que indivíduos com malária grave; e nenhuma diferença foi observada na resposta imune entre os participantes com malária não-complicada e aqueles com hiperbilirrubinemia. Ainda que o grupo de hiperbilirrubinemia tenha demonstrado um perfil menos inflamatório (que os casos graves), a resposta imune parece ainda ter um papel importante na patogênese desta condição. Nas análises por redes, observamos que IL-1β e IL-6 foram altamente conectadas a outros parâmetros inflamatórios, sugerindo que estas citocinas podem regular a qualidade da resposta imune nos indivíduos com hiperbilirrubinemia. Os níveis séricos de IL-6 já foram associados com desfechos graves da malária, e a hiperbilirrubinemia demonstrou ser um fator independentemente associado a concentrações elevadas desta molécula (DAY et al., 1999; KERN et al., 1989). Diversos estudos demonstraram que

os níveis de hepcidina estão aumentados nos casos de malária sintomática e assintomática, causados tanto pelo *P. falciparum* quanto pelo *P. vivax* (AYOYA et al., 2009; DE MAST et al., 2009a, 2009b, 2010; HOWARD et al., 2007). Nós encontramos que a hepcidina sérica está elevada nos indivíduos com malária grave e naqueles com malária e hiperbilirrubinemia comparados com os participantes com malária não-complicada. O aumento na hepcidina no grupo de hiperbilirrubinemia pode ser uma consequência do dano hepático, disfunção hepatocelular, e a resposta imune associada com esta condição. Recentemente, a hepcidina demonstrou ser o melhor preditor de incorporação de ferro pelos eritrócitos, o que sugere um papel deste hormônio em programas de suplementação de ferro (PRENTICE et al., 2012). Como descrevemos no estudo realizado, valores de *cut-off* da hepcidina podem ser usados para distinguir casos de malária grave daqueles com malária não-complicada (sem hiperbilirrubinemia) na área endêmica. De modo intrigante, alguns estudos têm mostrado que indivíduos com malária falciparum grave, por anemia grave ou malária cerebral, apresentam níveis baixos de hepcidina (BURTÉ et al., 2013; CASALS-PASCUAL et al., 2012; JONKER et al., 2013). Estas diferenças nas concentrações de hepcidina podem ser explicadas por diversos fatores: primeiro, estes estudos mensuraram a hepcidina em crianças, que apresentam uma anemia mais grave que os adultos (maioria dos casos graves do nosso trabalho); segundo, os níveis de citocinas nos casos de malária grave com baixa expressão de hepcidina estão mais baixos que nos casos de malária não-complicada, sugerindo a falta de estímulo inflamatório para a produção deste hormônio. No nosso estudo, por sua vez, indivíduos com malária grave tiveram uma

robusta resposta pró-inflamatória que pode estimular a expressão da hepcidina.

A hepcidina é regulada em resposta a diversas infecções e condições inflamatórias (SPOTTISWOODE; DUFFY; DRAKESMITH, 2014). Durante a infecção pelo plasmódio, IL-6 parece estar correlacionado com os níveis de hepcidina (ARMITAGE et al., 2009; BURTÉ et al., 2013; CASALS-PASCUAL et al., 2012; JONKER et al., 2013). Em camundongos, a produção de hepcidina durante a fase sanguínea da infecção previne uma infecção subsequente na fase hepática; e esta inibição foi preservada nos animais tratados com anticorpos anti-IL-6 (PORTUGAL et al., 2011). Em outro estudo, eritrócitos infectados pela malária induziram a síntese de RNAm de hepcidina por células mononucleares do sangue periférico, indicando a importância de células imunes circulatórias na produção deste hormônio (ARMITAGE et al., 2009). Recentemente, foi demonstrado que a IL-10 induz a expressão de hepcidina em macrofagos (HUANG et al., 2014). Nós observamos que a hepcidina foi positivamente correlacionada com a IL-6 e o IL-10 no grupo de malária não-complicada. Além disso, a hepcidina também foi positivamente correlacionada com o IFN-γ nos indivíduos com malária grave do nosso estudo, o que confirma o papel da resposta imune na regulação deste hormônio.

A Região Amazônica Brasileira é endêmica para a malária pelo *P. vivax* e para a infecção pelo vírus da dengue, com surtos epidêmicos destas doenças (MAGALHÃES et al., 2014). Não é comum a presença de indivíduos coinfectados pelo plasmódio e pela dengue; entretanto, a resposta laboratorial e imune desses pacientes permanecia pouco conhecida, sendo

um desafio para o manejo clínico. Em nosso próximo estudo, nós avaliamos a resposta imune (mensurada por citocinas e quimiocinas) e marcadores laboratoriais rotineiramente usados na prática clínica para entender melhor a imunopatologia dos casos de coinfecção malária e dengue.

Trabalhos prévios estudaram dosagens laboratoriais em indivíduos com coinfeção pela malária e dengue (ABBASI et al., 2009; EPELBOIN et al., 2012; MOHAPATRA; PATRA; AGRAWALA, 2012). Os valores de hemoglobina e plaquetas estão normalmente diminuídos em indivíduos com coinfecção *P. vivax* e dengue, e aspartato transaminase (AST) aumentado em indivíduos com malária comparados com os casos coinfectados (ABBASI et al., 2009; EPELBOIN et al., 2012; MOHAPATRA; PATRA; AGRAWALA, 2012). Estes achados são similares aos resultados encontrados em nosso estudo. Quando indivíduos apenas com dengue foram comparados com os casos de malária ou coinfecção em nosso trabalho, os primeiros tiveram concentrações maiores de hemoglobina e plaquetas. A infecção pelo plasmódio é frequentemente associada à anemia visto que este parasita tem uma fase sanguínea que causa intensa hemólise intravascular. Uma complicação frequentemente associada com ambas as doenças (dengue e malária) é a trombocitopenia, e os achados do nosso estudo indicam que a infecção pelo *P. vivax* pode causar uma trombocitopenia mais grave (com valores ainda mais baixos nos casos de coinfecção com a dengue) comparado com os casos de monoinfecção pelo vírus da dengue. Outros estudos também descrevem valores mais baixos de plaquetas em pacientes com malária do que indivíduos com dengue (KUTSUNA et al., 2014; SHAH; KATIRA, 2007). Baseado nos dados laboratoriais do nosso estudo, uma

curva ROC multi-paramétrica incluindo valores de hemoglobina, hematócrito, plaquetas, AST e ALT exibiu uma alta performance para distinguir os grupos (malária, dengue e coinfecção). Além disso, propomos um algoritmo simples de diagnóstico baseado nos valores de *cut-off* dos marcadores laboratoriais para discriminar a situação clínica dos indivíduos (Figura 4, publicada em nosso manuscrito). Neste algoritmo, indivíduos coinfectados podem ser distinguidos dos casos de monoinfecção pela dengue por níveis baixos de plaquetas e Hb, e daqueles com monoinfecção pela malária por níveis elevados e diminuídos de ALT e AST, respectivamente. Esses resultados, se validados em estudos maiores e em outras regiões endêmicas, podem fundamentar um algoritmo padronizado e validado para o uso clínico.

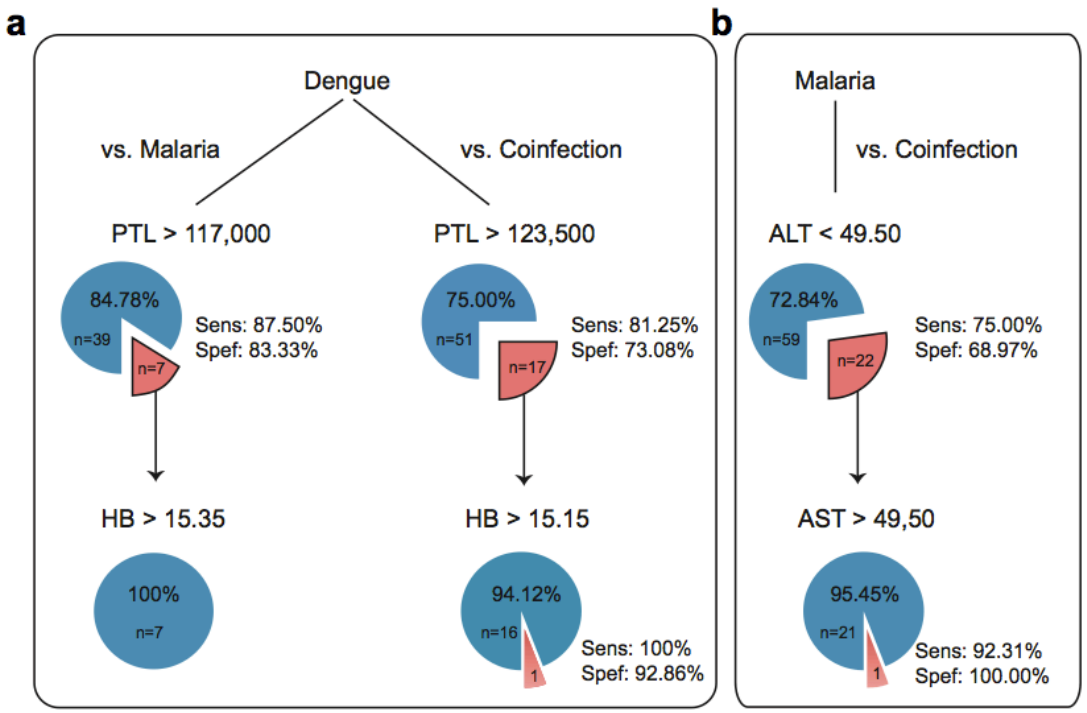


Figura 4. Algoritmo para diagnóstico de dengue, malária e casos de coinfecção através de dados laboratoriais. (Fonte: Mendonça et al. submetido)

Níveis plasmáticos elevados de TNF e IFN-γ têm sido sistematicamente associados ao aumento da gravidade clínica em malária ou dengue em diversas séries de casos (ANDRADE et al., 2010a; ARIAS et al., 2014; BOZZA et al., 2008; ESPINA et al., 2003; KURANE et al., 1991; LEVY et al., 2010). Papeis importantes do TNF e IFN-γ no desenvolvimento de sintomatologia na malária assim como nos processos patológicos associados com consumo de plaquetas, ativação endotelias e manifestações hemorrágicas na dengue têm sido descritas (ESPINA et al., 2003; GAGNON et al., 2002). No nosso estudo, níveis elevados de TNF foram observados nos participantes coinfectados comparados com as monoinfecções, e em associação com o número elevado de interações deste mediador com outras citocinas/quimiocinas sugerem que o TNF tem papel importante na patogênese da infecção simultânea entre dengue e malária. Adicionalmente, entre os grupos do estudo, os casos de coinfecção também exibiram os maiores valores das citocinas IFN-γ e IL-6. A IL-6 tem sido implicada na patogênese de casos graves de dengue visto que esta molécula estimula a produção de auto-anticorpos contra plaquetas e o endotélio vascular, assim como está envolvida na indução do ativador do plasminogênio tecidual, levando ao risco maior de sangramento (HUANG et al., 2003; RACHMAN; RINALDI, 2006). Nossos achados sobre marcadores imunológicos e laboratoriais apoiam a ideia de que os casos coinfectados podem ter uma inflamação e doença mais grave comparada com as monoinfecções pela dengue ou malária. Recentemente, foi descrito que grande parte dos indivíduos com infecção simultânea pela dengue e malária da Amazônia Brasileira e da Guiana Francesa exibiu uma doença clínica mais grave

comparada com os casos de infecção simples (EPELBOIN et al., 2012; MAGALHAES et al., 2012).

A malária vivax permanece um grande problema de saúde pública na Região Amazônica Brasileira com milhares de pessoas infectadas anualmente com número notório de óbitos, além do grande impacto socioeconômico para as comunidades endêmicas e para o sistema público de saúde. O conjunto de artigos desta Tese trazem dados que permitem o melhor entendimento dos fatores imunopatológicos de resistência e susceptibilidade associados com os diferentes desfechos clínicos da malária por *P. vivax*, que podem auxiliar futuros estudos intervencionais e de controle desta doença.

7. CONCLUSÕES

Os achados dos estudos que compõem a tese levam as seguintes conclusões:

- Polimorfismos genéticos nos eritrócitos, mediadores do sistema imune, e moléculas envolvidas em mecanismos de citoaderência influenciam o desfecho clínico da malária;

- Alterações genéticas e em níveis plasmáticos de moléculas envolvidas na via de metabolização do heme estão associadas com a malária vivax, sendo que indivíduos com o genótipo *Hp*2.2 e o componente longo da *HMOX1* assim como concentração elevada do sCD163 têm uma susceptibilidade maior de desenvolver malária sintomática;

- A análise por rede parece ser uma ferramenta útil para entender a interação entre diversos mediadores inflamatórios no contexto da malária vivax, a IL-4 parece ser uma molécula importante no grupo de malária assintomática devido ao grande número de conexões com outros marcadores;

- O alelo C do *DDX39B*-22C>G é um fator de risco para malária vivax complicada, diferentes haplótipos (incluindo os polimorfismos nos genes *DDX39B* e *TNF*) podem influenciar o desfecho clínico por alterar os níveis plasmáticos de TNF, e combinação de genótipos (incluindo o *IL6*-176G>C) estão associados com uma maior ou menor chance de

desenvolver malária clínica e podem influenciar os níveis séricos de TNF e IL-6;

- A concentração de citocinas e quimiocinas nos casos de malária com hiperbilirrubinemia é similar aos indivíduos com malária não-complicada e menos robusta que os casos graves, e os níveis de hepcidina estão aumentados nos casos de malária grave e malária com hiperbilirrubinemia, sendo que este hormônio está positivamente correlacionado com IL-6, IL-10 e IFN-γ nos diferentes desfechos clínicos da malária;

- Casos de coinfecção entre malária e dengue parecem desenvolver uma doença mais grave comparados às respectivas monoinfecções; estes indivíduos têm contagens mais diminuidas de plaquetas e concentracoes diminuidas de hemoglobina e apresentam uma bioassinatura imune específica com papel importante do TNF;

- A malária assintomática é um estado adquirido e ativo que controla a parasitemia e limita a disfunção orgânica; células imunes, especialmente células B e Treg, assim como biomarcadores da via do heme e do metabolismo do ferro parecem ter um papel importante nesta condição.

O conjunto de manuscritos desta Tese auxilia a compreensão da imunopatologia da malária vivax e podem auxiliar estudos futuros voltados para abordagens terapêuticas e de vacinas na Região Amazônica Brasileira.

8. REFERÊNCIAS

Aqui estão listadas as referências utilizadas na introdução e discussão geral da tese. As referencias citadas apenas nos manuscritos não estão listadas nesta seção.

ABBASI, A. et al. Clinical features, diagnostic techniques and management of dual dengue and malaria infection. **Journal of the College of Physicians and Surgeons--Pakistan : JCPSP**, v. 19, n. 1, p. 25–29, jan. 2009.

ABDEL-LATIF, M. S. et al. Antibodies to Plasmodium falciparum rifin proteins are associated with rapid parasite clearance and asymptomatic infections. **Infection and Immunity**, v. 71, n. 11, p. 6229–6233, nov. 2003.

ADEGNIKA, A. A.; KREMSNER, P. G. Epidemiology of malaria and helminth interaction: a review from 2001 to 2011. **Current Opinion in HIV and AIDS**, v. 7, n. 3, p. 221–224, maio 2012.

ALEXANDRE, M. A. et al. Severe Plasmodium vivax malaria, Brazilian Amazon. **Emerging Infectious Diseases**, v. 16, n. 10, p. 1611–1614, out. 2010.

ALLCOCK, R. J.; WILLIAMS, J. H.; PRICE, P. The central MHC gene, BAT1, may encode a protein that down-regulates cytokine production. **Genes to cells : devoted to molecular & cellular mechanisms**, v. 6, n. 5, p. 487–94, maio 2001.

ANAND, A. C.; PURI, P. Jaundice in malaria. **Journal of Gastroenterology and Hepatology**, v. 20, n. 9, p. 1322–1332, set. 2005.

ANDRADE, B. B. et al. Anti-Anopheles darlingi saliva antibodies as marker of Plasmodium vivax infection and clinical immunity in the Brazilian Amazon. **Malaria Journal**, v. 8, p. 121, jan. 2009.

ANDRADE, B. B. et al. Severe Plasmodium vivax malaria exhibits marked inflammatory imbalance. **Malaria Journal**, v. 9, p. 13, jan. 2010a.

ANDRADE, B. B. et al. Plasma superoxide dismutase-1 as a surrogate marker of vivax malaria severity. **PLoS Neglected Tropical Diseases**, v. 4, n. 4, p. e650, jan. 2010b.

ANDRADE, B. B. et al. Towards a precise test for malaria diagnosis in the Brazilian Amazon: comparison among field microscopy, a rapid diagnostic test, nested PCR, and a computational expert system based on artificial neural networks. **Malaria Journal**, v. 9, p. 117, jan. 2010c.

ANDRADE, B. B. et al. Heme impairs prostaglandin E2 and TGF-beta production by human mononuclear cells via Cu/Zn superoxide dismutase: insight into the pathogenesis of severe malaria. **Journal of Immunology,** v. 185, n. 2, p. 1196–204, 15 jul. 2010d.

ANDRADE, B. B. et al. Hepatitis B Infection Is Associated with Asymptomatic Malaria in the Brazilian Amazon. **PloS One**, v. 6, n. 5, p. e19841, jan. 2011.

ANDRADE, B. B.; BARRAL-NETTO, M. Biomarkers for susceptibility to infection and disease severity in human malaria. **Memórias do Instituto Oswaldo Cruz**, v. 106 Suppl, p. 70–78, ago. 2011.

ANSTEY, N. M. et al. Lung injury in vivax malaria: pathophysiological evidence for pulmonary vascular sequestration and posttreatment alveolar-capillary inflammation. **The Journal of Infectious Diseases**, v. 195, n. 4, p. 589–596, 15 fev. 2007.

ANTONELLI, L. R. V et al. The CD14+CD16+ inflammatory monocyte subset displays increased mitochondrial activity and effector function during acute Plasmodium vivax malaria. **PLoS Pathogens**, v. 10, n. 9, p. e1004393, set. 2014.

ARIAS, J. et al. Increased expression of cytokines, soluble cytokine receptors, soluble apoptosis ligand and apoptosis in dengue. **Virology**, v. 452-453, p. 42–51, mar. 2014.

ARMITAGE, A. E. et al. Plasmodium falciparum infected erythrocytes induce hepcidin (HAMP) mRNA synthesis by peripheral blood mononuclear cells. **British Journal of Haematology**, v. 147, n. 5, p. 769–771, dez. 2009.

ARRUDA, M. A. et al. Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide 3-kinase, MAPK, and NF-kappaB. **Journal of Immunology**, v. 173, n. 3, p. 2023–2030, 1 ago. 2004.

ASLEH, R. et al. Haptoglobin genotype- and diabetes-dependent differences in iron-mediated oxidative stress in vitro and in vivo. **Circulation Research**, v. 96, n. 4, p. 435–441, 4 mar. 2005.

ATKINSON, S. H. et al. Seasonal childhood anaemia in West Africa is associated with the haptoglobin 2-2 genotype. **PLoS Medicine**, v. 3, n. 5, p. e172, maio 2006.

ATKINSON, S. H. et al. The haptoglobin 2-2 genotype is associated with a reduced incidence of Plasmodium falciparum malaria in children on the coast of Kenya. **Clinical Infectious Diseases**, v. 44, n. 6, p. 802–809, 15 mar. 2007.

AYOYA, M. A. et al. Hepcidin and Plasmodium falciparum malaria in anemic school children in Mali. **Bulletin de la Société de Pathologie Exotique,** v. 102, n. 4, p. 219–220, out. 2009.

BACHTIAR, L. R. et al. Using artificial neural networks to classify unknown volatile chemicals from the firings of insect olfactory sensory neurons. Conference proceedings : In: ANNUAL INTERNATIONAL CONFERENCE OF THE IEEE ENGINEERING IN MEDICINE AND BIOLOGY SOCIETY. IEEE ENGINEERING IN MEDICINE AND BIOLOGY SOCIETY**. Annual Conference**, 2011. p. 2752–2755.

BACHTIAR, L. R.; UNSWORTH, C. P.; NEWCOMB, R. D. Application of artificial neural networks on mosquito Olfactory Receptor Neurons for an olfactory biosensor. Conference proceedings : In: ANNUAL INTERNATIONAL CONFERENCE OF THE IEEE ENGINEERING IN MEDICINE AND BIOLOGY SOCIETY. IEEE ENGINEERING IN MEDICINE AND BIOLOGY SOCIETY. Conference, 2013. p. 5390–5393.

BALLA, G. et al. Ferritin: a cytoprotective antioxidant strategem of endothelium. **The Journal of Biological Chemistry**, v. 267, n. 25, p. 18148–18153, 5 set. 1992.

BALLA, J. et al. Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. **Proceedings of the National Academy of Sciences of the United States of America**, v. 90, n. 20, p. 9285–9289, 15 out. 1993.

BALLA, J. et al. Ferriporphyrins and endothelium: a 2-edged sword-promotion of oxidation and induction of cytoprotectants. **Blood**, v. 95, n. 11, p. 3442–3450, 1 jun. 2000.

BAO, W. et al. Plasma heme oxygenase-1 concentration in relation to impaired glucose regulation in a non-diabetic Chinese population. **PloS One**, v. 7, n. 3, p. e32223, jan. 2012.

BARBOSA, S. et al. Epidemiology of disappearing Plasmodium vivax malaria: a case study in rural Amazonia. **PLoS Neglected Tropical Diseases**, v. 8, n. 8, p. e3109, ago. 2014.

BEJON, P. et al. A phase 2b randomised trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya. **PLoS Clinical Trials**, v. 1, n. 6, p. e29, jan. 2006.

BERBERAT, P. O. et al. Heavy chain ferritin acts as an antiapoptotic gene that protects livers from ischemia reperfusion injury. **FASEB Journal,** v. 17, n. 12, p. 1724–1726, set. 2003.

BERG, A. et al. Cytokine Network in Adults with Falciparum Malaria and HIV-1: Increased IL-8 and IP-10 Levels Are Associated with Disease Severity. **PloS One**, v. 9, n. 12, p. e114480, jan. 2014.

BERTIN, G. I. et al. Differential protein expression profiles between Plasmodium falciparum parasites isolated from subjects presenting with pregnancy-associated malaria and uncomplicated malaria in Benin. **The Journal of Infectious Diseases**, v. 208, n. 12, p. 1987–1997, 15 dez. 2013.

BLANN, A. D. et al. Increased levels of plasma haemoxygenase-1 in prostate cancer. **Prostate Cancer and Prostatic Diseases**, v. 14, n. 2, p. 114–117, jun. 2011.

BOUMA, G. et al. Secretion of tumour necrosis factor alpha and lymphotoxin alpha in relation to polymorphisms in the TNF genes and HLA-DR alleles. Relevance for inflammatory bowel disease. **Scandinavian Journal of Immunology**, v. 43, n. 4, p. 456–463, abr. 1996.

BOZZA, F. A. et al. Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. **BMC infectious diseases**, v. 8, p. 86, jan. 2008.

BRAGA, E. M. et al. Association of the IgG response to Plasmodium falciparum merozoite protein (C-terminal 19 kD) with clinical immunity to malaria in the Brazilian Amazon region. **The American Journal of Tropical Medicine and Hygiene**, v. 66, n. 5, p. 461–466, maio 2002.

BRULL, D. J. et al. Interleukin-6 gene -174g>c and -572g>c promoter polymorphisms are strong predictors of plasma interleukin-6 levels after coronary artery bypass surgery. **Arteriosclerosis, Thrombosis, and Vascular Biology**, v. 21, n. 9, p. 1458–1463, set. 2001.

BRUSIC, V. et al. Efficient discovery of immune response targets by cyclical refinement of QSAR models of peptide binding. **Journal of Molecular Graphics & Modelling**, v. 19, n. 5, p. 405–411, 467, jan. 2001
.

BURGNER, D. et al. Nucleotide and haplotypic diversity of the NOS2A promoter region and its relationship to cerebral malaria. **Human Genetics**, v. 112, n. 4, p. 379–386, abr. 2003.

BURTÉ, F. et al. Circulatory hepcidin is associated with the anti-inflammatory response but not with iron or anemic status in childhood malaria. **Blood**, v. 121, n. 15, p. 3016–3022, 11 abr. 2013.

CABANTOUS, S. et al. Genetic evidence for the aggravation of Plasmodium falciparum malaria by interleukin 4. **The Journal of Infectious Diseases**, v. 200, n. 10, p. 1530–1539, 15 nov. 2009.

CANDORE, G. et al. In vitro cytokine production by HLA-B8,DR3 positive subjects. **Autoimmunity**, v. 18, n. 2, p. 121–132, jan. 1994.

CARVALHO, L. H. et al. IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. **Nature Medicine**, v. 8, n. 2, p. 166–170, fev. 2002.

CASALS-PASCUAL, C. et al. Hepcidin demonstrates a biphasic association with anemia in acute Plasmodium falciparum malaria. **Haematologica**, 11 jun. 2012.

CHEN, T. et al. Elevated serum heme oxygenase-1 and insulin-like growth factor-1 levels in patients with Henoch-Schonlein purpura. **Rheumatology International**, v. 31, n. 3, p. 321–326, mar. 2011a.

CHEN, Y.-C. et al. Haptoglobin polymorphism as a risk factor for chronic kidney disease: a case-control study. **American Journal of Nephrology**, v. 33, n. 6, p. 510–514, jan. 2011b.

CHEN, Y.-H. et al. Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. **Human Genetics**, v. 111, n. 1, p. 1–8, jul. 2002.

CHERRY, C. L. et al. Cytokine genotype suggests a role for inflammation in nucleoside analog-associated sensory neuropathy (NRTI-SN) and predicts an individual's NRTI-SN risk. **AIDS Research and Human Retroviruses**, v. 24, n. 2, p. 117–123, fev. 2008.

CHIMMA, P. et al. A distinct peripheral blood monocyte phenotype is associated with parasite inhibitory activity in acute uncomplicated Plasmodium falciparum malaria. **PLoS Pathogens**, v. 5, n. 10, p. e1000631, out. 2009
.

CHIN, H. J. et al. The heme oxygenase-1 genotype is a risk factor to renal impairment of IgA nephropathy at diagnosis, which is a strong predictor of mortality. **Journal of Korean Medical Science**, v. 24 Suppl, p. S30–37, jan. 2009.

CLARK, T. G. et al. Tumor necrosis factor and lymphotoxin-alpha polymorphisms and severe malaria in African populations. **The Journal of Infectious Diseases**, v. 199, n. 4, p. 569–575, 15 fev. 2009.

COGSWELL, F. B. et al. Identification of hypnozoites and tissue schizonts of Plasmodium vivax and P. cynomolgi by the immunoperoxidase method. **The American Journal of Tropical Medicine and Hygiene**, v. 32, n. 6, p. 1454–1455, nov. 1983.

COHEN, S.; MCGREGOR, I. A.; CARRINGTON, S. Gamma-globulin and acquired immunity to human malaria. **Nature**, v. 192, p. 733–737, 25 nov. 1961.

COX, S. E. et al. Haplotype association between haptoglobin (Hp2) and Hp promoter SNP (A-61C) may explain previous controversy of haptoglobin and malaria protection. **PloS One**, v. 2, n. 4, p. e362, jan. 2007.

COZZI, A. et al. Overexpression of wild type and mutated human ferritin H-chain in HeLa cells: in vivo role of ferritin ferroxidase activity. **The Journal of Biological Chemistry**, v. 275, n. 33, p. 25122–9, 18 ago. 2000.

CRAMER, J. P. et al. iNOS promoter variants and severe malaria in Ghanaian children. **Tropical Medicine & International Health : TM & IH**, v. 9, n. 10, p. 1074–1080, out. 2004.

CUNHA, G. B. DA et al. Use of an artificial neural network to predict the incidence of malaria in the city of Cantá, state of Roraima. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 43, n. 5, p. 567–570, 2010.

DAUBERSIES, P. et al. Rapid turnover of Plasmodium falciparum populations in asymptomatic individuals living in a high transmission area. **The American Journal of Tropical Medicine and Hygiene**, v. 54, n. 1, p. 18–26, jan. 1996.

DAVENPORT, G. C. et al. Relationship between inflammatory mediator patterns and anemia in HIV-1 positive and exposed children with Plasmodium falciparum malaria. **American Journal of Hematology**, v. 87, n. 7, p. 652–658, jul. 2012.

DAY, K. P.; MARSH, K. Naturally acquired immunity to Plasmodium falciparum. **Immunology Today**, v. 12, n. 3, p. A68–71, mar. 1991.

DAY, N. P. et al. The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. **The Journal of Infectious Diseases**, v. 180, n. 4, p. 1288–1297, out. 1999.

DE LACERDA, M. V. G.; DE OLIVEIRA, S. L.; ALECRIM, M. DAS G. C. Splenic hematoma in a patient with Plasmodium vivax malaria. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 40, n. 1, p. 96–97, 2007.

DE MAST, Q. et al. Assessment of urinary concentrations of hepcidin provides novel insight into disturbances in iron homeostasis during malarial infection. **The Journal of Infectious Diseases**, v. 199, n. 2, p. 253–262, 15 jan. 2009a.

DE MAST, Q. et al. Mild increases in serum hepcidin and interleukin-6 concentrations impair iron incorporation in haemoglobin during an experimental human malaria infection. **British Journal of Haematology**, v. 145, n. 5, p. 657–664, jun. 2009b.

DE MAST, Q. et al. Increased serum hepcidin and alterations in blood iron parameters associated with asymptomatic P. falciparum and P. vivax malaria. **Haematologica**, v. 95, n. 7, p. 1068–1074, jul. 2010.

DEFORCHE, K. et al. Analysis of HIV-1 pol sequences using Bayesian Networks: implications for drug resistance. **Bioinformatics**, v. 22, n. 24, p. 2975–2979, 15 dez. 2006.

DEGLI-ESPOSTI, M. A.; LEELAYUWAT, C.; DAWKINS, R. L. Ancestral haplotypes carry haplotypic and haplospecific polymorphisms of BAT1: possible relevance to autoimmune disease. **European Journal of Immunogenetics,** v. 19, n. 3, p. 121–127, jun. 1992.

DEPINAY, N. et al. Inhibitory effect of TNF-α on malaria pre-erythrocytic stage development: influence of host hepatocyte/parasite combinations. **PloS One**, v. 6, n. 3, p. e17464, jan. 2011.

DOLO, H. et al. Filariasis attenuates anemia and proinflammatory responses associated with clinical malaria: a matched prospective study in children and young adults. **PLoS Neglected Tropical Diseases**, v. 6, n. 11, p. e1890, jan. 2012.

DORFMAN, J. R. et al. B cell memory to 3 Plasmodium falciparum blood-stage antigens in a malaria-endemic area. **The Journal of Infectious Diseases**, v. 191, n. 10, p. 1623–1630, 15 maio 2005.

DOUMBO, S. et al. Co-infection of long-term carriers of Plasmodium falciparum with Schistosoma haematobium enhances protection from febrile malaria: a prospective cohort study in Mali. **PLoS Neglected Tropical Diseases**, v. 8, n. 9, p. e3154, set. 2014.

DUNACHIE, S. J. et al. MIG and the regulatory cytokines IL-10 and TGF-β1 correlate with malaria vaccine immunogenicity and efficacy. **PloS One**, v. 5, n. 9, p. e12557, jan. 2010.

EPELBOIN, L. et al. Is dengue and malaria co-infection more severe than single infections? A retrospective matched-pair study in French Guiana. **Malaria Journal**, v. 11, p. 142, jan. 2012.

ESPINA, L. M. et al. Increased apoptosis and expression of tumor necrosis factor-alpha caused by infection of cultured human monocytes with dengue virus. **The American Journal of Tropical Medicine and Hygiene**, v. 68, n. 1, p. 48–53, jan. 2003.

EXNER, M. et al. The role of heme oxygenase-1 promoter polymorphisms in human disease. **Free Radical Biology & Medicine**, v. 37, n. 8, p. 1097–1104, 15 out. 2004.

FERREIRA, A. et al. A central role for free heme in the pathogenesis of severe malaria: the missing link? **Journal of Molecular Medicine**, v. 86, n. 10, p. 1097–111, out. 2008.

FERREIRA, A. et al. Sickle hemoglobin confers tolerance to Plasmodium infection. **Cell**, v. 145, n. 3, p. 398–409, 29 abr. 2011.

FLORI, L. et al. TNF as a malaria candidate gene: polymorphism-screening and family-based association analysis of mild malaria attack and parasitemia in Burkina Faso. **Genes and Immunity**, v. 6, n. 6, p. 472–480, set. 2005.

FRANCIS, S. E.; SULLIVAN, D. J.; GOLDBERG, D. E. Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. **Annual Review Of Microbiology**, v. 51, p. 97–123, jan. 1997.

FRANKENSTEIN, Z.; ALON, U.; COHEN, I. R. The immune-body cytokine network defines a social architecture of cell interactions. **Biology Direct**, v. 1, p. 32, jan. 2006.

FRIIS, H. et al. Iron, haptoglobin phenotype, and HIV-1 viral load: a cross-sectional study among pregnant Zimbabwean women. **Journal of Acquired Immune Deficiency Syndromes**, v. 33, n. 1, p. 74–81, 1 maio 2003.

GAGNON, S. J. et al. Cytokine gene expression and protein production in peripheral blood mononuclear cells of children with acute dengue virus infections. **Journal of Medical Virology**, v. 67, n. 1, p. 41–46, maio 2002.

GANZ, T. Hepcidin and its role in regulating systemic iron metabolism. **Hematology,** p. 29–35, jan. 2006.

GANZ, T. Hepcidin and iron regulation, 10 years later. **Blood**, v. 117, n. 17, p. 4425–33, 28 abr. 2011.

GETHING, P. W. et al. A long neglected world malaria map: Plasmodium vivax endemicity in 2010. **PLoS Neglected Tropical Diseases**, v. 6, n. 9, p. e1814, jan. 2012.

GIUSTI, P. et al. Plasmodium falciparum-infected erythrocytes and beta-hematin induce partial maturation of human dendritic cells and increase their migratory ability in response to lymphoid chemokines. **Infection and Immunity**, v. 79, n. 7, p. 2727–2736, jul. 2011.

GNJEC, A. et al. Association of alleles carried at TNFA -850 and BAT1 -22 with Alzheimer's disease. **Journal of neuroinflammation**, v. 5, p. 36, jan. 2008.

GONÇALVES, R. M. et al. CD4+ CD25+ Foxp3+ regulatory T cells, dendritic cells, and circulating cytokines in uncomplicated malaria: do different parasite species elicit similar host responses? **Infection and Immunity**, v. 78, n. 11, p. 4763–4772, nov. 2010.

GOZZELINO, R. et al. Metabolic Adaptation to Tissue Iron Overload Confers Tolerance to Malaria. **Cell Host & Microbe**, v. 12, n. 5, p. 693–704, 15 nov. 2012.

GOZZELINO, R.; JENEY, V.; SOARES, M. P. Mechanisms of cell protection by heme oxygenase-1. **Annual Review of Pharmacology and Toxicology**, v. 50, p. 323–354, jan. 2010.

GRAÇA-SOUZA, A. V et al. Neutrophil activation by heme: implications for inflammatory processes. **Blood**, v. 99, n. 11, p. 4160–4165, 1 jun. 2002.

HÖGGER, P. et al. Biochemical characterization of a glucocorticoid-induced membrane protein (RM3/1) in human monocytes and its application as model system for ranking glucocorticoid potency. **Pharmaceutical Research**, v. 15, n. 2, p. 296–302, fev. 1998.

HÖGGER, P.; SORG, C. Soluble CD163 inhibits phorbol ester-induced lymphocyte proliferation. **Biochemical and Biophysical Research Communications**, v. 288, n. 4, p. 841–843, 9 nov. 2001.

HOWARD, C. T. et al. Relationship of hepcidin with parasitemia and anemia among patients with uncomplicated Plasmodium falciparum malaria in Ghana. **The American Journal of Tropical Medicine and Hygiene**, v. 77, n. 4, p. 623–626, out. 2007.

HU, J. L. et al. Polymorphism in heme oxygenase-1 (HO-1) promoter and alcohol are related to the risk of esophageal squamous cell carcinoma on Chinese males. **Neoplasma**, v. 57, n. 1, p. 86–92, jan. 2010.

HUANG, H. et al. Interleukin-10 regulates hepcidin in Plasmodium falciparum malaria. **PloS One**, v. 9, n. 2, p. e88408, jan. 2014.

HUANG, Y.-H. et al. Tissue plasminogen activator induced by dengue virus infection of human endothelial cells. **Journal of Medical Virology**, v. 70, n. 4, p. 610–616, ago. 2003.

IMRIE, H. et al. Low prevalence of an acute phase response in asymptomatic children from a malaria-endemic area of Papua New Guinea. **The American Journal of Tropical Medicine and Hygiene**, v. 76, n. 2, p. 280–284, fev. 2007.

ISRAELSSON, E. et al. Cytokine gene haplotypes with a potential effect on susceptibility to malaria in sympatric ethnic groups in Mali. **Infection, Genetics and Evolution**, v. 11, n. 7, p. 1608–1615, out. 2011.

JACOB, C. O. et al. Heritable major histocompatibility complex class II-associated differences in production of tumor necrosis factor alpha: relevance to genetic predisposition to systemic lupus erythematosus. **Proceedings of the National Academy of Sciences of the United States of America**, v. 87, n. 3, p. 1233–1237, fev. 1990.

JAGANNATHAN, P. et al. IFNγ/IL-10 Co-producing Cells Dominate the CD4 Response to Malaria in Highly Exposed Children. **PLoS Pathogens**, v. 10, n. 1, p. e1003864, jan. 2014.

JANGPATARAPONGSA, K. et al. Immunity to malaria in Plasmodium vivax infection: a study in central China. **PloS One**, v. 7, n. 9, p. e45971, jan. 2012.

JENEY, V. et al. Pro-oxidant and cytotoxic effects of circulating heme. **Blood**, v. 100, n. 3, p. 879–887, 1 ago. 2002.

JOHN, C. C. et al. Low levels of RANTES are associated with mortality in children with cerebral malaria. **The Journal of Infectious Diseases**, v. 194, n. 6, p. 837–845, 15 set. 2006.

JONKER, F. A. M. et al. Low hepcidin levels in severely anemic malawian children with high incidence of infectious diseases and bone marrow iron deficiency. **PloS One**, v. 8, n. 12, p. e78964, jan. 2013.

JOOS, C. et al. Clinical protection from falciparum malaria correlates with neutrophil respiratory bursts induced by merozoites opsonized with human serum antibodies. **PloS one**, v. 5, n. 3, p. e9871, jan. 2010.

KARUNAWEERA, N. D. et al. Dynamics of fever and serum levels of tumor necrosis factor are closely associated during clinical paroxysms in Plasmodium vivax malaria. **Proceedings of the National Academy of Sciences of the United States of America**, v. 89, n. 8, p. 3200–3203, 15 abr. 1992.

KASSA, D. et al. Characterization of peripheral blood lymphocyte subsets in patients with acute Plasmodium falciparum and P. vivax malaria infections at Wonji Sugar Estate, Ethiopia. **Clinical and Vaccine Immunology : CVI**, v. 13, n. 3, p. 376–379, mar. 2006.

KASVOSVE, I. et al. Haptoglobin polymorphism and mortality in patients with tuberculosis. **The International Journal of Tuberculosis and Lung Disease,**, v. 4, n. 8, p. 771–775, ago. 2000.

KASVOSVE, I. et al. Effect of ferroportin Q248H polymorphism on iron status in African children. **The American Journal of Clinical Nutrition**, v. 82, n. 5, p. 1102–1106, nov. 2005.

KERN, P. et al. Elevated tumor necrosis factor alpha and interleukin-6 serum levels as markers for complicated Plasmodium falciparum malaria. **The American Journal Of Medicine**, v. 87, n. 2, p. 139–143, ago. 1989.

KIANG, R. et al. Meteorological, environmental remote sensing and neural network analysis of the epidemiology of malaria transmission in Thailand. **Geospatial Health**, v. 1, n. 1, p. 71–84, nov. 2006.

KILPINEN, S. et al. The promoter polymorphism of the interleukin-6 gene regulates interleukin-6 production in neonates but not in adults. **European Cytokine Network**, v. 12, n. 1, p. 62–68, mar. 2001.

KIMPARA, T. et al. Microsatellite polymorphism in the human heme oxygenase-1 gene promoter and its application in association studies with Alzheimer and Parkinson disease. **Human Genetics**, v. 100, n. 1, p. 145–147, jul. 1997.

KIRINO, Y. et al. Increased serum HO-1 in hemophagocytic syndrome and adult-onset Still's disease: use in the differential diagnosis of hyperferritinemia. **Arthritis Research & Therapy**, v. 7, n. 3, p. R616–24, jan. 2005.

KOCH, W. et al. Association of variants in the BAT1-NFKBIL1-LTA genomic region with protection against myocardial infarction in Europeans. **Human Molecular Genetics**, v. 16, n. 15, p. 1821–1827, 1 ago. 2007.

KOCHAR, D. K. et al. Severe Plasmodium vivax malaria: a report on serial cases from Bikaner in northwestern India. **The American Journal of Tropical Medicine and Hygiene**, v. 80, n. 2, p. 194–198, fev. 2009.

KOCHAR, D. K. et al. Clinical features of children hospitalized with malaria--a study from Bikaner, northwest India. **The American Journal of Tropical Medicine and Hygiene**, v. 83, n. 5, p. 981–989, nov. 2010.

KRISTIANSEN, M. et al. Identification of the haemoglobin scavenger receptor. **Nature**, v. 409, n. 6817, p. 198–201, 11 jan. 2001.

KROTOSKI, W. A. Discovery of the hypnozoite and a new theory of malarial relapse. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 79, n. 1, p. 1–11, jan. 1985.
KUESAP, J. et al. Study on association between genetic polymorphisms of haem oxygenase-1, tumour necrosis factor, cadmium exposure and malaria pathogenicity and severity. **Malaria Journal**, v. 9, p. 260, jan. 2010.

KURANE, I. et al. Activation of T lymphocytes in dengue virus infections. High levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2, and interferon-gamma in sera of children with dengue. **The Journal of Clinical Investigation**, v. 88, n. 5, p. 1473–1480, nov. 1991.

KUSI, K. A. et al. Levels of soluble CD163 and severity of malaria in children in Ghana. **Clinical and Vaccine Immunology : CVI**, v. 15, n. 9, p. 1456–1460, set. 2008.

KUTSUNA, S. et al. The usefulness of serum C-reactive protein and total bilirubin levels for distinguishing between dengue fever and malaria in returned travelers. **The American Journal of Tropical Medicine and Hygiene**, v. 90, n. 3, p. 444–448, mar. 2014.

KWIATKOWSKI, D. et al. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated Plasmodium falciparum malaria. **Lancet**, v. 336, n. 8725, p. 1201–1204, 17 nov. 1990.

KWIATKOWSKI, D. Genetic susceptibility to malaria getting complex. **Current Opinion in Genetics & Development**, v. 10, n. 3, p. 320–4, jun. 2000.

KWIATKOWSKI, D. P. How malaria has affected the human genome and what human genetics can teach us about malaria. **American Journal of Human Genetics**, v. 77, n. 2, p. 171–192, ago. 2005.

LACERDA, M. V. G. DE; HIPÓLITO, J. R.; PASSOS, L. N. DA M. Chronic Plasmodium vivax infection in a patient with splenomegaly and severe thrombocytopenia. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 41, n. 5, p. 522–523, 2008.

LACERDA, M. V. G. et al. Understanding the clinical spectrum of complicated Plasmodium vivax malaria: a systematic review on the contributions of the Brazilian literature. **Malaria Journal**, v. 11, p. 12, jan. 2012.

LEORATTI, F. M. DE S. et al. Neutrophil paralysis in Plasmodium vivax malaria. **PLoS Neglected Tropical Diseases**, v. 6, n. 6, p. e1710, jan. 2012.

LEVY, A. et al. Increment of interleukin 6, tumour necrosis factor alpha, nitric oxide, C-reactive protein and apoptosis in dengue. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 104, n. 1, p. 16–23, jan. 2010.

LINDBLADE, K. A. et al. The silent threat: asymptomatic parasitemia and malaria transmission. **Expert Review Of Anti-Infective Therapy**, v. 11, n. 6, p. 623–639, jun. 2013.

MAEDA, N. et al. Duplication within the haptoglobin Hp2 gene. **Nature**, v. 309, n. 5964, p. 131–135, 1984.

MAGALHAES, B. M. L. et al. Clinical Profile of Concurrent Dengue Fever and Plasmodium vivax Malaria in the Brazilian Amazon: Case Series of 11 Hospitalized Patients. **American Journal of Tropical Medicine and Hygiene**, v. 87, n. 6, p. 1119–1124, 1 out. 2012.

MAGALHÃES, B. M. L. et al. P. vivax Malaria and Dengue Fever Co-infection: A Cross-Sectional Study in the Brazilian Amazon. **PLoS Neglected Tropical Diseases**, v. 8, n. 10, p. e3239, out. 2014.

MARIKOVSKY, M. et al. Cu/Zn superoxide dismutase plays important role in immune response. **Journal of Immunology,** v. 170, n. 6, p. 2993–3001, 15 mar. 2003.

MCGUIRE, W. et al. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. **Nature**, v. 371, n. 6497, p. 508–510, 6 out. 1994.

MEDANA, I. M. et al. Cellular stress and injury responses in the brains of adult Vietnamese patients with fatal Plasmodium falciparum malaria. **Neuropathology and Applied Neurobiology**, v. 27, n. 6, p. 421–433, dez. 2001.

MEDZHITOV, R.; SCHNEIDER, D. S.; SOARES, M. P. Disease tolerance as a defense strategy. **Science,** v. 335, n. 6071, p. 936–941, 24 fev. 2012.

MELAMED-FRANK, M. et al. Structure-function analysis of the antioxidant properties of haptoglobin. **Blood**, v. 98, n. 13, p. 3693–3698, 15 dez. 2001.

MENDIS, K. et al. From malaria control to eradication: The WHO perspective. **Tropical Medicine & International Health : TM & IH**, v. 14, n. 7, p. 802–809, jul. 2009.

MEYER, C. G. et al. TNFalpha-308A associated with shorter intervals of Plasmodium falciparum reinfections. **Tissue \Antigens**, v. 59, n. 4, p. 287–292, abr. 2002.

MISHRA, S. K. et al. The influence of hyperbilirubinaemia on malaria-related mortality: an analysis of 1103 patients. **Annals of Tropical Medicine and Parasitology**, v. 98, n. 6, p. 555–558, set. 2004.

MIYAZAKI, T. et al. Serum HO-1 is useful to make differential diagnosis of secondary hemophagocytic syndrome from other similar hematological conditions. **International Journal of Hematology**, v. 91, n. 2, p. 229–237, mar. 2010.

MOHAPATRA, M. K.; PATRA, P.; AGRAWALA, R. Manifestation and outcome of concurrent malaria and dengue infection. **Journal of Vector Borne Diseases**, v. 49, n. 4, p. 262–265, dez. 2012.

MONTOSI, G. et al. Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. **The Journal of Clinical Investigation**, v. 108, n. 4, p. 619–623, ago. 2001.

MORDMÜLLER, B. G. et al. Tumor necrosis factor in Plasmodium falciparum malaria: high plasma level is associated with fever, but high production capacity is associated with rapid fever clearance. **European Cytokine Network**, v. 8, n. 1, p. 29–35, mar. 1997.

MORROT, A. et al. IL-4 receptor expression on CD8+ T cells is required for the development of protective memory responses against liver stages of malaria parasites. **The Journal of Experimental Medicine**, v. 202, n. 4, p. 551–560, 15 ago. 2005.

MS. **Portal da saúde: malária**. Disponível em: <http://portalsaude.saude.gov.br/index.php?option=com_content&view=article&id=10933&Itemid=646>. Acesso em: 17 dez. 2014.

NEMETH, E. et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. **The Journal of Clinical Investigation**, v. 113, n. 9, p. 1271–1276, maio 2004.

OHMORI, Y.; HAMILTON, T. A. STAT6 is required for the anti-inflammatory activity of interleukin-4 in mouse peritoneal macrophages. **The Journal of Biological Chemistry**, v. 273, n. 44, p. 29202–29209, 30 out. 1998.
OLIVEIRA, L. C. et al. Autoimmune hepatitis, HLA and extended haplotypes. **Autoimmunity reviews**, v. 10, n. 4, p. 189–93, fev. 2011.

OUMA, C. et al. Polymorphic variability in the interleukin (IL)-1beta promoter conditions susceptibility to severe malarial anemia and functional changes in IL-1beta production. **The Journal of Infectious Diseases**, v. 198, n. 8, p. 1219–1226, 15 out. 2008a.

OUMA, C. et al. Haplotypes of IL-10 promoter variants are associated with susceptibility to severe malarial anemia and functional changes in IL-10 production. **Human genetics**, v. 124, n. 5, p. 515–524, dez. 2008b.

PAMPLONA, A. et al. Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. **Nature medicine**, v. 13, n. 6, p. 703–710, jun. 2007.

PATH. **Staying the Course? Malaria Research and Development in a Time of Economic Uncertainty.** PATH ed. Seattle: [s.n.].

PINEDA-PEÑA, A.-C. et al. Trends and predictors of transmitted drug resistance (TDR) and clusters with TDR in a local Belgian HIV-1 epidemic. **PloS one**, v. 9, n. 7, p. e101738, jan. 2014.

PORTO, B. N. et al. Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors. **The Journal of Biological Chemistry**, v. 282, n. 33, p. 24430–24436, 17 ago. 2007.

PORTUGAL, S. et al. Host-mediated regulation of superinfection in malaria. **Nature medicine**, v. 17, n. 6, p. 732–7, jun. 2011.

PORTUGAL, S. et al. Exposure-dependent control of malaria-induced inflammation in children. **PLoS pathogens**, v. 10, n. 4, p. e1004079, abr. 2014.

POWRIE, F. et al. Control of immune pathology by regulatory T cells. **Novartis Foundation symposium**, v. 252, p. 92–8; discussion 98–105, 106–14, jan. 2003.

PRENTICE, A. M. et al. Hepcidin is the major predictor of erythrocyte iron incorporation in anemic African children. **Blood**, v. 119, n. 8, p. 1922–1928, 23 mar. 2012.

PRICE, P. et al. Polymorphisms at positions -22 and -348 in the promoter of the BAT1 gene affect transcription and the binding of nuclear factors. **Human Molecular Genetics**, v. 13, n. 9, p. 967–974, 1 maio 2004.

QUIÑONES-LOMBRAÑA, A. et al. BAT1 promoter polymorphism is associated with rheumatoid arthritis susceptibility. **The Journal of Rheumatology**, v. 35, n. 5, p. 741–744, maio 2008.

RACHMAN, A.; RINALDI, I. Coagulopathy in dengue infection and the role of interleukin-6. **Acta Medica Indonesiana**, v. 38, n. 2, p. 105–108, 2006.

RAHIMI, B. A. et al. Severe vivax malaria: a systematic review and meta-analysis of clinical studies since 1900. **Malaria journal**, v. 13, n. 1, p. 481, 8 dez. 2014.

RAMASAWMY, R. et al. BAT1, a putative anti-inflammatory gene, is associated with chronic Chagas cardiomyopathy. **The Journal of Infectious Diseases**, v. 193, n. 10, p. 1394–1399, 15 maio 2006.

ROBINSON, L. J. et al. Cellular tumor necrosis factor, gamma interferon, and interleukin-6 responses as correlates of immunity and risk of clinical Plasmodium falciparum malaria in children from Papua New Guinea. **Infection and Immunity**, v. 77, n. 7, p. 3033–3043, jul. 2009.

RODRIGUEZ-MORALES, A. J.; BENÍTEZ, J. A.; ARRIA, M. Malaria mortality in Venezuela: focus on deaths due to Plasmodium vivax in children. **Journal of Tropical Pediatrics**, v. 54, n. 2, p. 94–101, abr. 2008.

ROGERSON, S. What is the relationship between haptoglobin, malaria, and anaemia? **PLoS Medicine**, v. 3, n. 5, p. e200, maio 2006.

RONO, J. et al. Breadth of anti-merozoite antibody responses is associated with the genetic diversity of asymptomatic Plasmodium falciparum infections and protection against clinical malaria. **Clinical Infectious Diseases,** v. 57, n. 10, p. 1409–1416, nov. 2013.

SABCHAREON, A. et al. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. **The American Journal of Tropical Medicine and Hygiene**, v. 45, n. 3, p. 297–308, set. 1991.

SAMBO, M. R. et al. Transforming growth factor beta 2 and heme oxygenase 1 genes are risk factors for the cerebral malaria syndrome in Angolan children. **PloS One**, v. 5, n. 6, p. e11141, jan. 2010.
SÁNCHEZ-ARCILA, J. C. et al. Intestinal parasites coinfection does not alter plasma cytokines profile elicited in acute malaria in subjects from endemic area of Brazil. **Mediators of Inflammation**, v. 2014, p. 857245, jan. 2014.

SCHLUESENER, H. J.; KREMSNER, P. G.; MEYERMANN, R. Heme oxygenase-1 in lesions of human cerebral malaria. **Acta Neuropathologica**, v. 101, n. 1, p. 65–68, jan. 2001.

SEIXAS, E. et al. Heme oxygenase-1 affords protection against noncerebral forms of severe malaria. **Proceedings of the National Academy of Sciences of the United States of America**, v. 106, n. 37, p. 15837–15842, 15 set. 2009.

SENGUPTA, A. et al. Global host metabolic response to Plasmodium vivax infection: a 1H NMR based urinary metabonomic study. **Malaria Journal**, v. 10, p. 384, jan. 2011.

SHAH, I.; KATIRA, B. Clinical and laboratory profile of dengue, leptospirosis and malaria in children: a study from Mumbai. **Archives of Disease in Childhood**, v. 92, n. 6, p. 561, jun. 2007.

SHEU, C.-C. et al. Heme oxygenase-1 microsatellite polymorphism and haplotypes are associated with the development of acute respiratory distress syndrome. **Intensive Care Medicine**, v. 35, n. 8, p. 1343–13451, ago. 2009.

SHI, B. et al. Inferring Plasmodium vivax transmission networks from tempo-spatial surveillance data. **PLoS Neglected Tropical Diseases**, v. 8, n. 2, p. e2682, fev. 2014.

SIKORA, M. et al. A targeted association study of immunity genes and networks suggests novel associations with placental malaria infection. **PloS One**, v. 6, n. 9, p. e24996, jan. 2011.

SILVA, A. L. T. et al. Plasmodium vivax infection induces expansion of activated naïve/memory T cells and differentiation into a central memory profile. **Microbes and Infection / Institut Pasteur**, v. 15, n. 12, p. 837–843, nov. 2013.

SLATER, A. F.; CERAMI, A. Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. **Nature**, v. 355, n. 6356, p. 167–169, 9 jan. 1992.

SORTICA, V. A. et al. IL1B, IL4R, IL12RB1 and TNF gene polymorphisms are associated with Plasmodium vivax malaria in Brazil. **Malaria Journal**, v. 11, p. 409, jan. 2012.
SORTICA, V. A. et al. Role of IL6, IL12B and VDR gene polymorphisms in Plasmodium vivax malaria severity, parasitemia and gametocytemia levels in an Amazonian Brazilian population. **Cytokine**, v. 65, n. 1, p. 42–47, jan. 2014.

SPOTTISWOODE, N.; DUFFY, P. E.; DRAKESMITH, H. Iron, anemia and hepcidin in malaria. **Frontiers in Pharmacology**, v. 5, p. 125, jan. 2014.

STOCKER, R. et al. Bilirubin is an antioxidant of possible physiological importance. **Science,** v. 235, n. 4792, p. 1043–1046, 27 fev. 1987.

TAKEDA, M. et al. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to cerebral malaria in Myanmar. **Japanese Journal of Infectious Diseases**, v. 58, n. 5, p. 268–271, out. 2005.

TENHUNEN, R.; MARVER, H. S.; SCHMID, R. The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. **The Journal of Laboratory and Clinical Medicine**, v. 75, n. 3, p. 410–421, mar. 1970.

THORNTON, A. M.; SHEVACH, E. M. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. **Journal of Immunology,** v. 164, n. 1, p. 183–190, 1 jan. 2000.

TIMMERMANN, M.; HÖGGER, P. Oxidative stress and 8-iso-prostaglandin F(2alpha) induce ectodomain shedding of CD163 and release of tumor necrosis factor-alpha from human monocytes. **Free Radical Biology & Medicine**, v. 39, n. 1, p. 98–107, 1 jul. 2005.

TJITRA, E. et al. Multidrug-resistant Plasmodium vivax associated with severe and fatal malaria: a prospective study in Papua, Indonesia. **PLoS Medicine**, v. 5, n. 6, p. e128, 17 jun. 2008.

TOAN, N. L. et al. Co-infection of human parvovirus B19 with Plasmodium falciparum contributes to malaria disease severity in Gabonese patients. **BMC Infectious Diseases**, v. 13, p. 375, jan. 2013.

TRAN, T. M. et al. Systems immunology of human malaria. **Trends in Parasitology**, v. 28, n. 6, p. 248–257, jun. 2012.

TRAPE, J. F. et al. The Dielmo project: a longitudinal study of natural malaria infection and the mechanisms of protective immunity in a community living in a holoendemic area of Senegal. **The American Journal of Tropical Medicine and Hygiene**, v. 51, n. 2, p. 123–137, ago. 1994.

TRIPATHY, R. et al. Clinical manifestations and predictors of severe malaria in Indian children. **Pediatrics**, v. 120, n. 3, p. e454–460, set. 2007.

VERHOEFF, F. H. et al. Increased prevalence of malaria in HIV-infected pregnant women and its implications for malaria control. **Tropical Medicine & International Health : TM & IH**, v. 4, n. 1, p. 5–12, jan. 1999.

WAGENER, F. A. D. T. G. et al. HMOX1 promoter polymorphism modulates the relationship between disease activity and joint damage in rheumatoid arthritis. **Arthritis and Rheumatism**, v. 58, n. 11, p. 3388–3393, nov. 2008.

WALLACE, D. F. et al. Novel mutation in ferroportin1 is associated with autosomal dominant hemochromatosis. **Blood**, v. 100, n. 2, p. 692–694, 15 jul. 2002.

WALTHER, M. et al. Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. **Immunity**, v. 23, n. 3, p. 287–296, set. 2005.

WALTHER, M. et al. HMOX1 Gene Promoter Alleles and High HO-1 Levels Are Associated with Severe Malaria in Gambian Children. **PLoS Pathogens**, v. 8, n. 3, p. e1002579, mar. 2012.

WATTAVIDANAGE, J. et al. TNFalpha*2 marks high risk of severe disease during Plasmodium falciparum malaria and other infections in Sri Lankans. **Clinical and Experimental Immunology**, v. 115, n. 2, p. 350–355, fev. 1999.

WEAVER, L. K. et al. Pivotal advance: activation of cell surface Toll-like receptors causes shedding of the hemoglobin scavenger receptor CD163. **Journal of Leukocyte Biology**, v. 80, n. 1, p. 26–35, jul. 2006.

WENISCH, C. et al. Plasma levels of the interleukin-6 cytokine family in persons with severe Plasmodium falciparum malaria. **The Journal of Infectious Diseases**, v. 179, n. 3, p. 747–750, mar. 1999.

WERE, T. et al. Suppression of RANTES in children with Plasmodium falciparum malaria. **Haematologica**, v. 91, n. 10, p. 1396–1399, out. 2006.

WERE, T. et al. Naturally acquired hemozoin by monocytes promotes suppression of RANTES in children with malarial anemia through an IL-10-dependent mechanism. **Microbes and Infection / Institut Pasteur**, v. 11, n. 8-9, p. 811–819, 2009.

WHITE, N. J. The role of anti-malarial drugs in eliminating malaria. **Malaria journal**, v. 7 Suppl 1, p. S8, jan. 2008.

WHITWORTH, J. et al. Effect of HIV-1 and increasing immunosuppression on malaria parasitaemia and clinical episodes in adults in rural Uganda: a cohort study. **Lancet**, v. 356, n. 9235, p. 1051–1056, 23 set. 2000.

WHO. WHO | Guidelines for the treatment of malaria. Second edition. 2010.

WHO. WHO | World Malaria Report 2014. 2014.

WILSON, A. G. et al. An allelic polymorphism within the human tumor necrosis factor alpha promoter region is strongly associated with HLA A1, B8, and DR3 alleles. **The Journal of Experimental Medicine**, v. 177, n. 2, p. 557–560, 1 fev. 1993.

YAMADA, N. et al. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. **American Journal of Human Genetics**, v. 66, n. 1, p. 187–195, jan. 2000.

9. ANEXO I: PRODUÇÃO CIENTÍFICA ADICIONAL

Artigos produzidos durante o período do doutorado e que não entraram no corpo da tese:

MENDONÇA, V. R. R. ; ALCANTARA, T. ; ANDRADE, N. ; ANDRADE, B. B. ; BARRAL-NETTO, M. ; OLIVEIRA, V. S. B. . Analysis of theoretical knowledge and the practice of science among brazilian otorhinolaryngologists. Brazilian Journal of Otorhinolaryngology (Impresso), v. 79, p. 487-493, 2013.

COSTA, A. S. A. ; COSTA, G. C. ; AQUINO, D. M. C. ; **MENDONÇA, V. R. R.** ; BARRAL, A. ; BARRAL-NETTO, M. ; CALDAS, A. J. M. . Cytokines and visceral leishmaniasis: a comparison of plasma cytokine profiles between the clinical forms of visceral leishmaniasis. Memórias do Instituto Oswaldo Cruz (Impresso), v. 107, p. 735-739, 2012.

MENDONÇA, V. R. R. ; ANDRADE, B. B. ; ALMEIDA, A. ; BARRAL-NETTO, M. . Can Score Databanks Help Teaching?. Plos One, v. 6, p. e15695, 2011.

MENDONÇA, V. R. R. ; ANDRADE, B. B. ; ALMEIDA, A. ; BARRAL-NETTO, M. . Can Score Databanks Help Teaching?. Plos One, v. 6, p. e15695, 2011.
MENDONÇA, V. R. R. ; BARRAL-NETTO, M. . Stimulating the formation of the physician-scientist. Scientific exposure during the medical course in Brazil. Medical Science Educator, v. 21, p. 107-111, 2011.

ANDRADE, B. B. ; SANTOS, C. J. N. ; CAMARGO, L. M. ; SOUZA-NETO, S. M. ; REIS-FILHO, A. ; CLARENCIO, J. ; **MENDONÇA, V. R. R.** ; LUZ, N. F. ; CAMARGO, E. P. ; BARRAL, A. ; SILVA, A. A. M. ; BARRAL-NETTO, M. . Hepatitis B Infection Is Associated with Asymptomatic Malaria in the Brazilian Amazon. Plos One, v. 6, p. e19841, 2011.

LIMA, D. S. C. ; FIGUEREDO, ; ROCHA, E. A. ; COSTA, V. H. M. V. ; CASTRO, M. P. ; SILVA, R. C. P. ; CHAGAS, G. L. ; ARAUJO, L. P. ; **MENDONÇA, V. R. R.** ; GRAVINA, P. R. ; MENESES, J. V. L. . Anatomical study of the mandibular foramen and its relation with reference points of the mandibular ramus. Revista da Sociedade Brasileira de Cirurgia Craniomaxilofacial, v. 14, p. 91-96, 2011.

Artigos produzidos antes do início do doutorado:

REIS-FILHO, A. ; ANDRADE, B. B. ; **MENDONÇA, V. R. R.** ; BARRAL-NETTO, M. . Research knowledge in undergraduate school in Brazil: a comparison between medical and law students. Einstein (São Paulo), v. 8, p. 273-280, 2010.

LIMA, D. S. C. ; FIGUEREDO, A. A. ; GRAVINA, P. R. ; **MENDONÇA, V. R. R.** ; CASTRO, M. P. ; CHAGAS, G. L. ; ARAUJO, L. P. ; SILVA, R. C. P. ; COSTA, V. H. M. V. ; ROCHA, E. A. ; MENESES, J. V. L. . Anatomic characterization of mental foramen in a sample of Brazilian's human dry mandibles. Revista da Sociedade Brasileira de Cirurgia Craniomaxilofacial, v. 13, p. 230-235, 2010.