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

FIOCRUZ

Curso de Pós-graduação em Patologia Humana

TESE DE DOUTORADO

IDENTIFICAÇÃO DE POTENCIAIS DETERMINANTES IMUNOLÓGICOS DE GRAVIDADE DA MALÁRIA HUMANA

Bruno de Bezerril Andrade

Salvador – Bahia – Brasil
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Bruno de Bezerril Andrade

Orientador: Manoel Barral Netto

Tese apresentada ao Colegiado do Curso de Pós-graduação em Patologia Humana, como pré-requisito obrigatório para obtenção do grau Doutor.

Salvador – Bahia – Brasil
2010

"Identificação de potenciais determinantes imunológicos de gravidade da malária humana"

Bruno de Bezerril Andrade

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LISTA DE ABREVIATURAS

CD4	Co-receptor de linfócito T auxiliar
CD8	Co-receptor de linfócito T citotóxico
CD14	Co-receptor associado a alguns receptores do tipo toll
CD25	Receptor da interleucina 2
GPI	Glicofosfatidilinositol
IFN	Interferon
IL	Interleucina
MalDANN	"Malaria Diagnosis Based on Artificial Neural Networks", software para o diagnóstico de malária baseado em redes neurais artificiais.
PCR	Reação em cadeia de polimerase
PfEMP	Proteína da superfície de eritrócitos infectados por *P. falciparum*
PGE2	Prostraglandina E_2
SOD-1	Cu/Zn Superóxido dismutase, superóxido dismutase-1
TGF	Fator transformador de crescimento
Th	Linfócito T auxiliar
TLR4	Receptor do tipo toll 4
TNF	Fator de necrose tumoral
TNK	Células T "Natural Killers", apresentam características mistas de células T e de células matadoras naturais
VHB	Vírus da Hepatite B

ANDRADE, Bruno Bezerril. Identificação de potenciais determinantes imunológicos de gravidade na malária humana. Tese (Doutorado) – Centro de Pesquisas Gonçalo Moniz, Salvador, Bahia, 2010.

RESUMO

A malária é considerada uma das mais importantes doenças infecciosas do mundo. Esta doença é causada por diversas espécies do protozoário *Plasmodium sp.*, principalmente o *Plasmodium falciparum* e o *Plasmodium vivax*, transmitido por mosquitos do gênero *Anopheles*. Apesar dos esforços governamentais e privados para o desenvolvimento de estratégias para o controle da doença, o panorama atual da malária está piorando, muito em razão do aparecimento de cepas de parasitas resistentes aos medicamentos. Os casos fatais são relatados principalmente na África e são causados pelo *Plasmodium falciparum*. Apesar de ser menos letal, a malária causada pelo *Plasmodium vivax* é mais amplamente distribuída e pode apresentar também alta morbidade e mortalidade. Na maioria das áreas endêmicas, estudos têm identificado vários fatores relacionados à imunidade clínica ou susceptibilidade aos parasitas. Assim, pelo menos quanto à malária causada pelo *Plasmodium falciparum*, idade, polimorfismos genéticos e exposição repetida ao parasita são considerados importantes determinantes da evolução da doença. Infelizmente, pouco tem sido feito na identificação de fatores preditores consistentes que poderiam ser usados para avaliação clínica. Este quadro é ainda pior para malária causada pelo *Plasmodium vivax*, provavelmente porque muitos pesquisadores consideram que é uma doença benigna. Além disso, como a maioria do conhecimento atual sobre a patogênese da malária não ajudou a reduzir a ocorrência da infecção e suas complicações, novas abordagens são necessárias para superar este cenário desfavorável. Esta Tese reúne um conjunto de seis manuscritos que visam identificar potenciais determinantes da gravidade da malária em uma área endêmica da Amazônia Ocidental Brasileira. Em primeiro lugar, um método preciso e eficaz para o diagnóstico da malária foi rastreado através da comparação de vários testes, incluindo um software baseado em redes neurais artificiais. O ensaio molecular mostrou-se o mais eficiente para o diagnóstico da malária sintomáticos e assintomáticos. Além disso, a utilização racional de um teste rápido para diagnóstico da malária pode ser promissora em áreas onde há dificuldade na formação continuada dos técnicos diagnósticos. A rede neural artificial indicou que o balanço de citocinas é um forte determinante do quadro clínico. Em outro estudo, uso de sorologia para mensuração de anticorpos IgG contra o sonicado de glândula salivar do vetor *Anopheles darlingi* mostrou-se útil para a avaliação da exposição ao *Plasmodium vivax* e também para estimar a imunidade clínica à malária. Em um terceiro estudo com foco na identificação de outros fatores relacionados à imunidade clínica, a exposição natural ao vírus da hepatite B mostrou-se associada à redução da gravidade clínica da malária causada tanto pelo *Plasmodium vivax* quanto pelo *Plasmodium falciparum*. No que diz respeito exclusivamente à malária vivax, os casos graves apresentaram uma intensa e desregulada resposta inflamatória sistêmica. Nestes pacientes, a enzima antioxidante superóxido dismutase-1 surgiu como um excelente marcador da gravidade e mostrou-se envolvida na patogênese da doença grave, na qual há uma liberação de grandes quantidades de heme livre. Em conjunto, os manuscritos desta tese adicionam importantes informações no entendimento dos mecanismos determinantes da gravidade da malária, extremamente úteis no direcionamento de futuras abordagens focadas no controle da doença.

Palavras-chave: Malaria, diagnóstico, biomarcador, inflamação, citocina.

ANDRADE, Bruno Bezerril. Identification of potential immunologic determinants of severity in human malaria. Tese (Doutorado) – Centro de Pesquisas Gonçalo Moniz, Salvador, Bahia, 2010.

ABSTRACT

Malaria is considered one of the most important infectious diseases that ever threaten the world. This disease is caused mainly by the infection with *Plasmodium falciparum* or *Plasmodium vivax* transmitted by *Anopheles* mosquitoes. Despite governmental and private efforts for the development of key strategies for the disease control, the actual panorama of the *Plasmodium* infection is getting worse due to the emergence of drug resistant parasite strains. The lethal cases are reported mostly in Africa and are caused by *Plasmodium falciparum*. Albeit being less lethal, *Plasmodium vivax* infections are more widely distributed can cause high morbidity and eventually death. In most endemic areas, studies have indentified a number of factors related to clinical immunity or susceptibility to the parasites. Thus, at least regarding the falciparum malaria, age, genetic polymorphisms and repeated exposure to *Plasmodium* are considered most important determinants of the disease outcome. Unfortunately, little has been made in the screening of reliable predicting factors that could be ultimately used for clinical evaluations. This landscape is even worse for vivax malaria, probably because many researches consider it as a benign disease. Moreover, as most of the current knowledge about the malaria pathogenesis did not truly help to relieve the disease burden, new insights are necessary to overcome this unfavorable scenario. This thesis brings together a set of six manuscripts that aim to identify potential determinants of the disease severity linked to the immunopathogenesis in an endemic area from the western Brazilian Amazon. First, a precise and effective method for malaria diagnosis was screening by comparing multiple tests, including a software based of artificial neural networks. The molecular assay showed to be the most efficient for the diagnosis of symptomatic and asymptomatic malaria. In addition, the rational use of a rapid test for the diagnosis of malaria may be promising in areas where there is difficulty in continued training of technical human resources. The artificial neural network indicated that the cytokine balance is a strong determinant of the clinical presentation. In another study, the use of serology for measuring IgG antibodies against the sonicate salivary gland of *Anopheles darlingi* vector is a promising marker of exposure to *Plasmodium vivax* and can also estimate the clinical immunity. Intriguingly, the natural exposure to the hepatitis B virus appeared as an important factor associated with reduced clinical severity for both vivax and falciparum malaria. Concerning solely the vivax malaria, severe cases have an intense and unregulated inflammatory response. In these patients, the antioxidant enzyme superoxide dismutase-1 has emerged as an excellent marker of severity and was involved in the pathogenesis of the severe disease in which there is a release of large amounts of free heme. Together, the manuscripts of this thesis add important information in understanding the mechanisms that determine the severity of malaria.

Keywords: Malaria, diagnosis, biomarker, inflammation, cytokine.

LISTA DOS ARTIGOS

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

Manuscrito I

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.

Manuscrito II

Anti-*Anopheles darlingi* saliva antibodies as marker of *Plasmodium vivax* infection and clinical immunity in the Brazilian Amazon.

Malar J 2009, 8:121.

Manuscrito III

Hepatitis B infection reduces malaria severity.

Clinical Infectious Diseases 2010 (submetido)

Manuscrito IV

Severe *Plasmodium vivax* malaria exhibits marked inflammatory imbalance.

Malar J 2010, 9:13.

Manuscrito V

Plasma superoxide dismutase-1 as a surrogate marker of vivax malaria severity.

PLoS Negl Trop Dis 2010, 4(4):e650.

Manuscrito VI

Heme impairs PGE_2 and TGF-β1 production by human mononuclear cells via Cu/Zn superoxide dismutase: insight into the pathogenesis of severe malaria.

J Immunol 2010, 15;185(2):1196-204.

1. INTRODUÇÃO

1.1 A MALÁRIA COMO IMPORTANTE PROBLEMA DE SAÚDE PÚBLICA

A malária humana é uma doença infecciosa, não contagiosa, com manifestações clínicas episódicas de caráter agudo. É causada por protozoários do gênero *Plasmodium* e transmitida ao homem na natureza através da picada de mosquitos do gênero *Anopheles*. Acomete aproximadamente 500 milhões de pessoas e causa de 1,5 a 2,7 milhões de óbitos por ano, sendo que quase 34% da população mundial vive em áreas onde há risco de transmissão da doença (WHO, 2008). É prevalente em mais de 100 países, porém, mais de 90% dos casos ocorrem na África Sub-Saariana (WHO, 2008). Excluindo os países africanos, 2/3 dos casos concentram-se apenas em seis países: Índia, Brasil, Sri Lanka, Afeganistão, Vietnã e Colômbia. No continente americano, Brasil, Peru e Colômbia contribuem com 70% dos registros da doença (WHO, 2008).

No Brasil, a malária incide fundamentalmente na Amazônia legal (divisão política do território nacional que engloba nove Estados: Amazonas, Pará, Acre, Roraima, Rondônia, Amapá, Mato Grosso, Tocantins, e Maranhão) (Brasil, 2008). Somente nesta região, a malária registra aproximadamente 500 mil casos por ano, com um aumento de 26% entre 2003 e 2006 (Brasil, 2008). Destacam-se pela intensidade de transmissão os Estados do Pará, Amazonas e Rondônia, responsáveis por 80% dos casos relatados (Brasil, 2008). Em termos gerais, a malária no Brasil é considerada hipoendêmica/mesoendêmica e a transmissão é instável, com flutuações sazonais ocorrendo durante o ano (Camargo *et al.*, 1996). Entretanto, existem áreas

em que a taxa de transmissão apresenta-se elevada (Rodrigues Ade *et al.*, 2008). Devido a sua alta incidência e seus fatores debilitantes, a malária é a doença que mais contribui para a decadência do homem na região amazônica, reduzindo a qualidade de vida e atuando como fator limitante do crescimento demográfico, cultural e econômico. As infecções maláricas no território brasileiro não apresentam mortalidade tão elevada como na África, mas interferem significativamente a capacidade laborativa e o bem estar da população que vive nas áreas endêmicas, constituindo um enorme problema de saúde pública.

1.2 OS AGENTES ETILÓGICOS, OS VETORES E O CICLO BIOLÓGICO

Existem algumas espécies de parasitas que naturalmente infectam o homem: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* e *Plasmodium ovale*. Recentes estudos relataram casos de infecções pelo *Plasmodium knowlesi* em humanos, em sua maioria Ásia (Daneshvar *et al.*, 2009; Van Den Eede *et al.*, 2009). O *P. vivax* é a espécie mais amplamente distribuída pelas zonas tropicais e subtropicais do globo, especialmente na Ásia, América do Sul e Oceania (Guerra *et al.*, 2006). O *P. falciparum* é mais prevalente na África, existindo também em algumas regiões das Américas e do sudoeste asiático. No Brasil, a maior parte dos casos de malária é devida ao *P. vivax* (76% dos registros), no entanto, é constatado nas estatísticas um incremento do percentual de malária por *P. falciparum* (Brasil, 2008). É possível a infecção de um mesmo paciente por mais de uma espécie de plasmódio, conhecida como "forma mista". No Brasil, as formas mistas são quase sempre a associação entre *P. vivax* e *P. falciparum*. A prevalência de infecções por *P. malariae* é baixa, mas

alguns estudos sugerem que pode alcançar 10% em algumas regiões (Cavasini *et al.*, 2000). Não há malária causada pelo *P. ovale* no Brasil.

Há muitas espécies de mosquitos *Anopheles spp.* no Brasil (Deane, 1986; Galardo *et al.*, 2009), mas dentre as diversas espécies, o vetor *Anopheles (An.) darling* é o de maior importância epidemiológica pela sua grande distribuição no território nacional, pelo alto grau de antropofilia e endofagia, estando perfeitamente adaptado ao ecossistema amazônico, o que dificulta seu controle e erradicação (Deane, 1986). Nas áreas endêmicas, os moradores são expostos intensamente a picadas destes e outros mosquitos. Entretanto, a taxa de infecção dos insetos vetores geralmente varia de 0,1% a 10% (Lines *et al.*, 1991; Gil *et al.*, 2003). Assim, cada habitante é exposto muito mais aos insetos não infectados do que os infectados. Tem-se sugerido a partir de estudos experimentais que a exposição continuada a picadas de *An. darlingi* não infectados poderiam modificar a resposta imunológica do hospedeiro contra o plasmódio (Donovan *et al.*, 2007).

A infecção inicia-se quando esporozoítos infectantes são inoculados no homem pelo inseto vetor, dando início ao ciclo pré-eritrocitário, clinicamente silencioso. Há uma rápida migração para o fígado, onde os os esporozoítos invadem os hepatócitos e se desenvolvem em esquizontes que se multiplicam assexuadamente. O *P. vivax* e o *P. ovale* podem evoluir neste momento para uma fase estacionária denominada hipnozoíto que pode permanecer latente durante meses e até anos, dando origem aos esquizontes teciduais em um período variável de tempo, responsáveis pelas recidivas da doença (Cogswell *et al.*, 1983; Krotoski, 1985). O determinante

biológico que influencia a evolução para o esquizonte replicante ou o hipnozoíto é desconhecido, assim como o processo que leva à ativação do hipnozoíto. Após alguns ciclos de replicação, os esquizontes hepáticos se rompem e liberam milhares de merozoítos na corrente sanguínea.

Na fase eritrocitária, os merozoítos liberados dos hepatócitos invadem eritrócitos e se desenvolvem em trofozoítos, os quais se multiplicam e maturam, formando novos esquizontes que se rompem liberando mais merozoítos, com nova invasão de eritrócitos. O ciclo sanguíneo se repete sucessivas vezes, a cada 48 horas nas infecções por *P. vivax* e *P. falciparum* (Greenwood *et al.*, 2005). A cada ciclo eritrocitário, a liberação de diversas substâncias tóxicas, como o heme livre, enzimas líticas, radicais livres e a hemozoína que é metabolizada pelo plasmódio (Coban *et al.*, 2005; Awandare *et al.*, 2007; Hanscheid *et al.*, 2008), induz estresse oxidativo, inflamação e ativação do sistema imunológico. É nesta fase da infecção em que usualmente aparecem os sintomas. Nesta fase do ciclo, algumas diferenças biológicas entre o *P. vivax* e o *P. falciparum* são importantes determinantes da gravidade da doença. O *P. vivax* preferencialmente infecta reticulócitos (Anstey *et al.*, 2009). A base biológica desta aparente predileção não é conhecida, além da descrição de ligantes específicos para a invasão celular (Galinski *et al.*, 1992), como os antígenos glicoprotéicos Duffy (Miller *et al.*, 1976). Isso também poderia representar uma adaptação do parasita para evitar hiperparasitemias e doença mais grave, ou mesmo o fato de que os reticulócitos poderiam oferecer um microambiente especial para o crescimento parasitário. Conseqüentemente, as infecções por *P. vivax* geralmente apresentam reduzida parasitemia e desfechos clínicos menos graves quando comparados aos casos de malária causados por *P. falciparum*. Outra diferença

importante é que o *P. vivax* torna-se muito mais amebóide do que o *P. falciparum* enquanto está se desenvolvendo dentro dos reticulócitos, causando maior deformabilidade (Suwanarusk *et al.*, 2004). Todas as formas do *P. vivax* são encontradas na circulação periférica, como a maioria das espécies de plasmódio (exceto o *P. falciparum*), e pode ser que a deformabilidade aumentada auxilie o parasita na passagem pelo baço. Se isso for verdade, o *P. vivax* não precisaria de propriedades adesivas para o seqüestro nos capilares periféricos a fim de escapar do sistema retículo endotelial. Entretanto, a escassez de citoaderência e seqüestro periférico na infecção pelo *P. vivax* precisa de uma reavaliação, em razão de evidências de que pode ocorrer aderência no baço (Del Portillo *et al.*, 2004) e no pulmão (Anstey *et al.*, 2007). Este cenário contrasta com a consistência ausência de formas sexuais maduras nas infecções por *P. falciparum*, exceto nos casos graves com alta parasitemia (Miller *et al.*, 1994). Isso se deve ao fato deste parasita ser capaz de aderir a uma variedade de receptores endoteliais e ficar e ficar seqüestrado no leito capilar de vários tecidos e órgãos (Miller *et al.*, 1994). As hemácias infectadas pelas formas maduras do *P. falciparum* são rígidas e facilmente capturadas pelo sistema retículo endotelial. Esta característica peculiar do *P. falciparum* provavelmente traz implicações importantes para a ocorrência de formas graves, como a malária cerebral.

Alguns merozoítos resultantes da esquizogonia eritrocitária se diferenciam em gametócitos, que responsáveis pela infecção do mosquito durante o repasto sanguíneo. Aqui aparece mais uma diferença importante entre as duas mais prevalentes espécies de plasmódio. Os gametócitos do *P. vivax* desenvolvem-se precocemente durante a infecção e podem ser vistos na circulação periférica no começo ou mesmo pouco antes do início dos sintomas (Boyd e Kitchen, 1937). Desta

maneira, indivíduos com malária assintomática que não receberam ainda tratamento podem servir como reservatórios e transmitir o parasita para os mosquitos vetores (Alves *et al.*, 2005). Esta transmissão antes do aparecimento da doença e tratamento pode explicar em parte a resistência à terapia anti-malárica surgiu mais de 30 anos depois dos primeiros relatos para o *P. falciparum*. Assim, a maioria dos gametócitos produzidos durante a infecção em tese teriam sido expostos menos terapia, reduzindo a chance da transmissão de cepas mutantes resistentes. Apesar de tentadora, esta idéia previamente aceita pelos malariologistas tem que ser revista, já que uma análise retrospectiva da concentração sanguínea de gametócitos em pacientes experimentalmente infectados com *P. vivax* mostrou que estes não são vistos no sangue antes do início dos sintomas (Mckenzie *et al.*, 2007).

1.3 A SITUAÇÃO ATUAL DO DIAGNOSTICO DA MALÁRIA

Nas diversas áreas endêmicas de malária, as quais predominam em países pobres ou em desenvolvimento, o diagnóstico da infecção ainda carece de melhorias. Nessas áreas, é comum haver alta prevalência de muitas outras doenças infecciosas que apresentam quadro clínico semelhante à malária, como a febre amarela, dengue e leptospirose, o que pode trazer confundimento no diagnóstico diferencial. A ineficiência diagnóstica certamente correlaciona-se com o retardo no acesso ao tratamento adequado e portanto aumento da morbidade e mortalidade. Apesar dos avanços tecnológicos, o método da visualização microscópica dos parasitas utilizando esfregaços sanguíneos é ainda considerado o padrão ouro a ser aplicado nas áreas endêmicas. O diagnóstico parasitológico utilizando a microscopia requer supervisão e treinamento continuado de pessoal, além de uma estrutura laboratorial mínima, o que

é difícil manter em áreas remotas de desertos e florestas. Além disso, há uma considerável variação da eficácia diagnóstica relacionada à experiência do técnico microscopista (Coleman, Maneechai, Rachaphaew *et al.*, 2002; Bowers *et al.*, 2009; Alexander *et al.*, 2010).

Outros métodos diagnósticos foram desenvolvidos para tentar melhorar o panorama do diagnóstico da malária. Há mais de dez anos, métodos baseados em amplificação de material genético por reação em cadeia de polimerase (PCR) foram padronizados para o diagnóstico de malária (Snounou *et al.*, 1993; Snounou, 1996). Os métodos que utilizam tanto nested-PCR quanto PCR em tempo real apresentam maior sensibilidade e especificidade do que a microscopia, principalmente quanto à identificação de casos com baixa parasitemia (Di Santi *et al.*, 2004; Costa *et al.*, 2008; Shokoples *et al.*, 2009). Apesar disso, os ensaios moleculares são custosos e requerem mais investimento em infra-estrutura do que a microscopia, o que reduz a sua aplicabilidade nas áreas endêmicas. Assim, o uso de ensaios moleculares atualmente restringe-se ao campo de pesquisa.

Uma promissora aquisição no campo do diagnóstico da malária foram os testes rápidos (rapid diagnostic test, RDT). A maioria destes testes utilizam imunocromatografia para identificação de produtos parasitários (Murray *et al.*, 2008). Tais testes são mais baratos do que os moleculares, não carecem de estrutura laboratorial, são facilmente aplicados, apresentam leitura simples e rápida e podem apresentar sensibilidade e especificidade equivalentes à microscopia bem realizada (Ashley *et al.*, 2009; Valea *et al.*, 2009). Entretanto, a maioria dos estudos de validação destes testes foi realizada em países que apresentam alta endemicidade

(Tjitra *et al.*, 1999; Coleman, Maneechai, Ponlawat *et al.*, 2002; Coleman, Maneechai, Rachapaew *et al.*, 2002; Pattanasin *et al.*, 2003), sendo necessárias portanto mais investigações em áreas de variável prevalência. O diagnóstico preciso feito através de ferramentas inovadoras eficazes e de baixo custo são fundamentais para a identificação precoce dos casos da infecção e portanto são também essenciais para o manejo adequado e controle da transmissão.

1.4 A APRESENTAÇÃO CLÍNICA DA DOENÇA E A RESPOSTA IMUNE DO HOSPEDEIRO

Em todo o mundo, a maioria das infecções por plasmódios é clinicamente silenciosa, refletindo a habilidade dos mecanismos imunológicos adaptativos em prevenir os sintomas (Greenwood *et al.*, 2005). Em indivíduos não-imunes, contudo, as infecções são clinicamente mais evidentes, e uma minoria de casos pode se tornar grave, com acometimento de múltiplos órgãos e causar a morte. A manifestação clínica da doença depende da espécie do plasmódio, da idade e da imunidade anti-malárica do hospedeiro (Druilhe e Perignon, 1997; 1998).

Em áreas de alto risco de transmissão (incidência de 70 casos por 1.000 habitantes), crianças, gestantes e indivíduos provenientes de áreas não endêmicas (migrantes ou visitantes ocasionais) compõem o grupo de pacientes mais propensos a desenvolver a doença, representada basicamente por crises febris periódicas, anemia, acidose metabólica e malária cerebral. Entretanto, em áreas de baixo risco (incidência de 0,1 casos por 1.000 habitantes), a infecção primária geralmente ocorre em adultos,

nos quais a doença grave freqüentemente envolve distúrbios adicionais, tais como disfunção hepática, insuficiência renal, edema pulmonar e choque (Schofield e Grau, 2005). Estas manifestações mais graves da infecção, em geral, correlacionam-se com o nível de parasitemia. Há hiperparasitemia quando mais de 2% das hemácias do hospedeiro primo-infectado estão parasitadas ou mais de 5% nos indivíduos que já tiveram malária no passado (Brasil, 2005). Na prática, consideram-se hiperparasitados os pacientes que apresentam, ao exame da gota espessa, positividade igual ou superior a três cruzes ou presença de esquizontes com qualquer nível de parasitemia. Enfim, a dinâmica da transmissão e a idade do hospedeiro, assim como o seus perfis genético e imunológico são importantes determinantes da doença. É importante notar que a maioria dos estudos neste escopo referem-se a infecções causadas pelo *P. falciparum*, devido a alta letalidade. O conhecimento sobre os fatores determinantes da gravidade da malária causada pelo *P. vivax* ainda é escasso e apenas recentemente tem recebido devida atenção (Anstey *et al.*, 2009; Mueller *et al.*, 2009). Antes considerada uma infecção relativamente benigna quando comparada à infecção pelo *P. falciparum*, a malária causada pelo *P. vivax* tem sido recentemente associada com complicações graves e morte (Price *et al.*, 2007).

A suscetibilidade humana à infecção malárica parece ser universal. Porém, há fatores inatos, não diretamente imunológicos, relacionados à proteção natural à infecção. No caso do *P. vivax*, a ausência de iso-antígenos do sistema sanguíneo Duffy impede a penetração dos merozoítos nas hemácias (Miller *et al.*, 1976). Este achado entretanto está sendo rediscutido frente aos recentes relatos de infecções por *P. vivax* em indivíduos Duffy negativos na África (Ryan *et al.*, 2006) e no Brasil (Cavasini *et al.*, 2007). Já a presença da hemoglobina S nos portadores do traço

falcêmico, assim como a deficiência genética de glicose-6-fosfato-desidrogenase, reduz a gravidade da infecção por *P. falciparum* (Greenwood *et al.*, 2005).

A diversidade das síndromes parece confundir a determinação de um mecanismo unificador da patogênese da malária. Apesar de já existirem inúmeros estudos em humanos, a maioria do conhecimento sobre a imunopatogênese da doença ainda resulta de estudos experimentais. Há indícios de que a interseção do poucos processos básicos pode determinar as diversas síndromes: a localização específica de eritrócitos parasitados em órgãos-alvo; a ação local e sistêmica de produtos bioativos do parasita, como toxinas, nos tecidos do hospedeiro; a produção local e sistêmica e citocinas e quimiocinas pró-inflamatórias e contra-regulatórias pelo sistema imunológico inato e adaptativo em resposta aos produtos do parasita; e o recrutamento e ativação de células inflamatórias, com ação da imunidade celular e humoral (Schofield e Grau, 2005). De acordo com este ponto de vista, as diversas síndromes clínicas que sucedem à infecção malárica são o estágio final do processo de ativação de cascatas inflamatórias atípicas e respostas imunológicas inadequadas à eliminação do plasmódio.

Evidências sugerem que anticorpos e células T apresentam papel crucial na imunidade protetora contra as diferentes formas evolutivas do plasmódio (Good *et al.*, 1998). Anticorpos contra moléculas da superfície dos merozoítos freiam o ciclo eritrocitário através do bloqueio da invasão de novas hemácias (Giha *et al.*, 2000). Está bem estabelecido que anticorpos, principalmente do isotipo IgG, direcionados contra antígenos do *P. falciparum* na fase eritrocitária são importantes na imunidade

anti-malárica, sendo que a transferência do soro de uma pessoa imune para outra não-imune garante um efeito protetor (Mcgregor, 1964). Entretanto, a função das subclasses de IgG na aquisição da imunidade anti-malárica é ainda incerta. Respostas humorais também atuam contra esporozoítos, inibindo sua invasão nos hepatócitos (Hisaeda *et al.*, 2005) ou interferindo a ligação de eritrócitos infectados no endotélio, como os anticorpos anti-PfEMP1 (proteína da superfície de eritrócitos infectados por *P. falciparum*, responsável pelo seqüestro de hemácias na microcirculação) (Giha *et al.*, 2000). Além disso, anticorpos específicos contra moléculas de glicosilfosfatidilinositol (GPI), uma das moléculas imuno-estimulatórias, podem suprimir a ativação de macrófagos, resultando em uma menor produção de citocinas inflamatórias e menor patologia (Schofield *et al.*, 2002). Hospedeiros também desenvolvem anticorpos anti-gametócitos, que interferem na transmissão de parasitas aos mosquitos vetores. Apesar deste tipo de imunidade não proteger indivíduos infectados, pode ajudar a reduzir a infecção no nível da comunidade, embora este mecanismo pareça ser insignificante na prática (Hisaeda *et al.*, 2005).

A resposta imune celular anti-malárica parece ser de fundamental importância para o controle da parasitemia, porém, paradoxalmente, encontra-se também envolvida com o estabelecimento da doença grave. Células T CD8+ exibem atividade citotóxica contra hepatócitos parasitados, limitando parcialmente a maturação dos esporozoítos (Good *et al.*, 1998). Se a liberação de merozoítos do fígado para a corrente sanguínea é prevenida, a infecção poderia ser controlada antes do aparecimento da doença clínica, porém, na prática, a imunidade da fase pré-eritrocitária mostra-se pouco eficaz frente aos mecanismos de escape do plasmódio. Células T CD4+ são indispensáveis para a proteção contra parasitas da fase

eritrocitária, seja através da assistência à produção de anticorpos neutralizantes e opsonizantes, seja através da liberação de citocinas pró-inflamatórias para ativação de macrófagos e estimulação da eliminação de hemácias infectadas (Good *et al.*, 1998). Dados de estudos clínicos e experimentais indicam que uma resposta inflamatória precoce, com a produção de interleucina (IL)-1, IL-2, interferon (IFN)-gama e fator de necrose tumoral (TNF)-alfa, é requerida para o controle inicial da multiplicação intra-eritrocitária dos parasitas (Stevenson *et al.*, 1995; Fell e Smith, 1998); resistência é absolutamente dependente de IFN-gama (Favre *et al.*, 1997), e a falha da manutenção das respostas iniciais Th1 pode levar ao aumento da carga parasitária. IFN-gama e TNF- alfa atuam sinergicamente para induzir a destruição de parasitas dentro de células fagocitárias, concentradas no baço (Favre *et al.*, 1997). Entretanto, em excesso, as citocinas pró-inflamatórias são as maiores favorecedoras da doença grave (Clark *et al.*, 2006; Clark *et al.*, 2008). No homem, o risco de morte por malária cerebral correlaciona-se com altas concentrações de TNF- alfa (Grau *et al.*, 1989). A mortalidade em adultos está associada com altos níveis séricos de IL-6 e IL-10 (Day *et al.*, 1999). A anemia grave em crianças está ligada a altas concentrações de TNF-alfa e baixas concentrações de IL-10 (Kurtzhals *et al.*, 1998; Othoro *et al.*, 1999). A indução de fator de transformação de crescimento (TGF)-beta *in vitro* está associada a um risco reduzido de doença grave (Dodoo *et al.*, 2002). Por outro lado, outros estudos evidenciaram que concentrações mais elevadas de IL-10 estão relacionadas a uma redução de dano tecidual, incluindo a malaria experimental (Kossodo *et al.*, 1997) e humana (Ho *et al.*, 1998). A otimização da resposta imune anti-malárica, então, depende de um fino ajuste do balanço de citocinas pró-inflamatórias e anti-inflamatórias. Neste sentido, a razão IFN-gama/IL-10 tem sido utilizada para estimar o balanço pró-inflamatório nos pacientes com malaria (Metenou *et al.*, 2009).

Indivíduos que vivem em áreas endêmicas da malária eventualmente desenvolvem imunidade clínica (Druilhe e Perignon, 1997) e anti-parasita, descrita como espécie-específica, cepa-específica e estágio-específica (Hisaeda *et al.*, 2005). Entretanto, a imunidade adquirida não é completa, não sendo capaz de erradicar o protozoário, mas pode limitar a carga parasitária a níveis extremamente baixos e reduzir drasticamente a gravidade dos sintomas e complicações da doença, muitas vezes tornando o hospedeiro assintomático (Druilhe e Perignon, 1997). Esta imunidade, conhecida como "premunição", é adquirida lenta e progressivamente após estímulos antigênicos constantes, determinados por infecções repetidas pelo mesmo parasita (*P. falciparum* ou *P. vivax*), o que explica em parte a observação de que os indivíduos das áreas endêmicas acometidos pela doença clínica são representados, em sua maioria, por crianças e adultos jovens, que não tiveram tempo suficiente para desenvolver este estado imunitário. A "premunição" é um fenômeno lábil e geralmente desaparece após seis meses de ausência completa do estímulo antigênico (Druilhe e Perignon, 1997). Isto acontece quando cessam as re-infecções após o abandono da área endêmica pelo paciente. Além disso, esta imunidade costuma ser perdida nas gestantes (Hisaeda *et al.*, 2005).

Diversas são as explicações possíveis para a incapacidade do organismo em desenvolver uma resposta imune esterilizante, como normalmente ocorre em infecções virais e bacterianas. Os complexos mecanismos de escape dos plasmódios podem responder algumas questões, mas necessitam de melhor elucidação. O plasmódio é um parasita intracelular, sendo capaz de suprimir a expressão de

moléculas do complexo principal de histocompatibilidade (MHC)-II e dificultar a resposta celular citotóxica (Hisaeda *et al.*, 2005). O parasita também induz modificação da superfície de hemácias, facilitando a aderência ao endotélio vascular e dificultando a eliminação de parasitas pelo sistema retículo-endotelial. Outros mecanismos são a diversidade de antígenos entre as várias formas evolutivas do parasita, o polimorfismo antigênico entre as cepas e a variação antigênica clonal de uma mesma cepa, que tornam as respostas imunes específicas menos eficazes. Por fim, os plasmódios são capazes de causar imunossupressão específica, interferindo na maturação de células dendríticas e induzindo proliferação de células imunorregulatórias, tais como linfócitos CD4+ CD25+ e células T NK (Hisaeda *et al.*, 2004). A ativação de células imunorregulatórias correlaciona-se com a produção de citocinas imunomodulatórias, como o TGF-beta e IL-10, supressoras da resposta pró-inflamatória (Shevach, 2002). Células com características regulatórias são rapidamente induzidas após a infecção de eritrócitos por parasitas e são associadas a um pico precoce de produção de TGF-beta, diminuição da produção de citocinas pró-inflamatórias e a uma redução das respostas imunes antígenos-específicas (Hisaeda *et al.*, 2004). Tanto a produção precoce de TGF-beta quanto a presença de células T regulatórias estão associadas a maiores taxas de crescimento parasitário *in vivo* (Hisaeda *et al.*, 2004). A indução de células T regulatórias mediada pelo *P. falciparum* deve representar um fator de virulência do parasita. Entretanto, ao mesmo tempo em que beneficiam o parasita, facilitando o estabelecimento da infecção, as células T regulatórias induzidas pelo plasmódio podem contribuir para o controle das respostas inflamatórias, em um momento mais tardio da infecção, reduzindo a imunopatologia e prevenindo a malária grave.

O fenômeno da "premunição" é classicamente descrito em áreas africanas de alta endemicidade, e em infecções por *P. falciparum* (Druilhe e Perignon, 1997). Entretanto, a descrição de uma alta prevalência de malária assintomática no Brasil demonstra que a resistência adquirida também ocorre neste país, como relatado na África, a despeito da diferença epidemiológica da doença entre tais países (Alves *et al.*, 2002). Estudos desenvolvidos na Amazônia mostraram que a imunidade naturalmente adquirida contra o *P. vivax* também existe e parece ser induzida mais rapidamente do que a imunidade anti-*P. falciparum* (Camargo, E. P. *et al.*, 1999).

As conseqüências clínicas da malária assintomática ainda não são totalmente compreendidas. Ao passo que é aceito amplamente que, em áreas endêmicas, a malária assintomática está envolvida no desenvolvimento da imunidade parcial (Druilhe e Perignon, 1997) e deve proteger contra a doença grave em casos de novas infecções, os pacientes assintomáticos não são incluídos nos esquemas atuais de tratamento e podem servir de reservatório para a transmissão da doença nas áreas endêmicas. Um levantamento epidemiológico sobre a malária no Estado de Rondônia no Brasil, através da revelação de um significativo número de indivíduos com malária assintomática, sugeriu que são estes indivíduos, e não os imigrantes, os maiores reservatórios epidemiológicos para a transmissão contínua de malária (Camargo, L. M. *et al.*, 1999). Estudos na África, por sua vez, sugeriram que os indivíduos assintomáticos parecem ser incomuns e freqüentemente evoluem para a doença clínica, sugerindo que o tratamento destes pacientes pode ser benéfico, em termos de prevenção da malária grave (Owusu-Agyei *et al.*, 2002). Entretanto, um outro estudo africano evidenciou que o tratamento de casos assintomáticos pode aumentar o risco de ocorrência da malária sintomática em crianças quando estas são re-infectadas após

terapia anti-malárica adequada (Njama-Meya *et al.*, 2004). Após a re-infecção, os pacientes anteriormente portadores de malária assintomática (tratados) apresentaram parasitemias mais baixas e sintomas mais amenos da doença, mas demonstraram uma taxa de re-infecção semelhante à dos pacientes sintomáticos tratados (Njama-Meya *et al.*, 2004).

Estudos experimentais reforçam a noção de que células T regulatórias podem reduzir a imunopatologia da malária, sendo que tais células e suas citocinas imunomodulatórias seriam as grandes responsáveis pela malária assintomática. Baixas concentrações séricas de TGF-beta estão associadas à doença aguda e grave e um desequilíbrio entre os níveis sistêmicos de citocinas pró-inflamatórias e TGF-beta aumenta o risco da doença (Omer e Riley, 1998). Assim, em casos de malária moderada, uma vez que a parasitemia está sob controle, células T regulatórias devem produzir ou induzir a produção de TGF-beta (Omer e Riley, 1998) e IL-10 (Li *et al.*, 2003), os quais modulam a resposta inflamatória. Indivíduos nos quais a resposta das células T regulatórias é defeituosa devem ter risco aumentado de progredir para a malária grave. Além disso, através da inibição dos mecanismos efetores persistentes mediados pela resposta Th1, a atividade da célula T regulatória deve favorecer a persistência da malária assintomática, favorecendo tanto a memória imunológica adquirida (premunição) quanto a transmissão parasitária em áreas endêmicas.

Apesar da importante contribuição dos estudos experimentais no entendimento da patogênese da malária, o desfecho clínico da malária humana parece originar de relações mais complexas. Dentre os múltiplos fatores que interferem na resposta

imunológica do hospedeiro humano contra o plasmódio, as co-infecções provavelmente desenvolvem um importante papel. Muitas outras infecções são comuns nas áreas endêmicas de malária. Dentre estas destacam-se as infecções virais (Braga *et al.*, 2005; Bronzan *et al.*, 2007; Karp e Auwaerter, 2007) e as helmintíases (Yatich *et al.*; Nkuo-Akenji *et al.*, 2006; Nacher, 2008). Estas infecções concomitantes interferem na resposta imunológica do hospedeiro e podem trazer vantagens ou desvantagens em relação a gravidade da apresentação clínica da malária. Alguns relatos associam hepatite viral com pior prognóstico na malária grave (Thursz *et al.*, 1995; Barcus *et al.*, 2007) e as infecções helmínticas podem exacerbar a malária (Helmby, 2009) ou reduzir a sua imunopatologia (Lyke *et al.*, 2005; Brutus *et al.*, 2007; Metenou *et al.*, 2009). Essas associações precisam ser melhor exploradas e os mecanismos responsáveis pela modulação das respostas carecem de melhor entendimento.

Certamente são inúmeros os fatores que podem se associar à apresentação clínica da doença. Ao mesmo tempo que é tentador avaliar os determinantes da imunidade clínica, a investigação sobre os fatores envolvidos na patogênese da doença grave é fundamental importância para o entendimento da malária e do embasamento para futuras intervenções profiláticas e terapêuticas.

2. JUSTIFICATIVA

Apesar da queda no numero de casos a partir de 2006, a malária no Brasil continua a ser um grande problema de saúde pública, exercendo uma enorme carga econômica ao sistema único de saúde (SUS) no Brasil. Os Estados do Pará, Amazonas, Rondônia e Mato Grosso apresentam os mais numerosos registros de casos. A estratégia de combate à doença realizada pelo Ministério da Saúde baseia-se em ações preconizadas pela Organização Mundial da Saúde, e inclui o combate ao vetor, o diagnóstico precoce e preciso e o tratamento otimizado. Apesar de tais medidas serem bem sucedidas no controle do alastramento de casos, a política de controle de malária não é capaz de erradicar a doença sem o auxílio de medidas inovadoras. Esta tese é composta de seis sub-estudos que investigaram quatro importantes aspectos da malária no Brasil: o diagnóstico de casos assintomáticos, a estimativa de exposição ao vetor, a influência de co-infecções na apresentação clínica da infecção e o entendimento de complicações na malária vivax. O nosso grupo de pesquisa identificou tais problemas cujas soluções seriam fundamentais para auxiliar o combate a esta infecção e gerar queda nos custos de saúde. O primeiro grande problema é o do diagnóstico da malária assintomática. Estudos prévios realizados no Brasil mostraram que os indivíduos com malária assintomática podem permanecer longos períodos com o plasmódio, e são também capazes de transmitir o parasita para vetores não infectados. Isto sugere que os indivíduos com malária assintomática podem servir de reservatórios da doença. Nós idealizamos um programa baseado em redes neurais artificiais capaz de predizer a malária assintomática através da coleta de informações epidemiológicas e antropométricas. Caso seja validado, tal software poderia ser usado em áreas endêmicas estratégicas para tratamento sistemático de

casos, visando a eliminação da transmissão. Para a identificação destas áreas, seria necessário estimar as regiões de risco de exposição ao vetor. O grupo então padronizou e validou uma técnica de sorologia para estimar tal exposição. Futuramente, estas duas ferramentas em conjunto podem ser de grande valia em um programa de erradicação. Raros são os estudos no Brasil que avaliam a influência de co-infecções na apresentação clínica da malária. Nós decidimos avaliar o impacto da hepatite viral B na malária porque as áreas de distribuição destas doenças no mundo são coincidentes. Isto é importante para orientar políticas de controle de doenças simultâneas. Recentemente, casos atípicos de malária vivax com maior morbidade e letalidade têm sido relatados no Brasil. É de fundamental importância caracterizar tais casos e identificar ferramentas de predição do diagnóstico, além de identificar possíveis alvos terapêuticos. Nosso grupo descreve biomarcadores dos casos graves e testa o estresse oxidativo como alvo terapêutico, para o qual já há drogas para uso em humanos. Em conjunto, a tese traz valorosas contribuições para o entendimento da malária no Brasil. A descrição de determinantes de proteção ou gravidade são fundamentais para guiar futuros esquemas de manejo dos pacientes ou do controle epidemiológico.

3. OBJETIVOS

Identificar determinantes do diagnóstico preciso da malária humana e descrever potenciais candidatos a biomarcadores de proteção ou gravidade com base na imunopatogênese da doença, em uma área endêmica da Amazônia brasileira.

3.1 OBJETIVOS ESPECÍFICOS

- Estabelecer uma metodologia eficaz para rastreamento diagnóstico de casos de malária sintomática e assintomática em uma amostra de pacientes para servir como base de análise dos determinantes imunológicos.

- Padronizar e validar o uso da mensuração sorológica de anticorpos anti-saliva do vetor *Anopheles darlingi* para estimar exposição e imunidade clínica ao *Plasmodium vivax*.

- Investigar a associação entre infecção pelo vírus da hepatite B e a apresentação clínica da malária.

- Descrever o perfil epidemiológico, inflamatório e imunológico da malária grave causada pelo *Plasmodium vivax*.

- Identificar candidatos biomarcadores de gravidade clínica da malária vivax humana que apresentem íntima relação com a imunopatogênese da doença.

4. METODOLOGIA

Resumo geral da metodologia empregada para a amostragem dos indivíduos estudados na série de manuscritos

732 pessoas foram recrutadas entre 2006 e 2007, em Buritis e em comunidades ribeirinhas de Porto Velho, duas regiões de Rondônia, Estado que apresenta alta incidência de malária. Para recrutamento dos casos foram utilizadas: busca passiva, com atendimento nos postos da Fundação Nacional da Saúde, postos municipais e no Hospital Municipal de Buritis; e busca ativa, com rastreamento domiciliar em regiões onde estudos prévios revelaram alta prevalência de casos, incluindo assintomáticos. Após consentimento assinado, uma entrevista e exame físico, coleta de sangue e o exame da gota espessa foram realizados. Os dados dos pacientes foram registrados em um banco de dados digitalizado. O sangue foi utilizado para o diagnóstico molecular da malária (*nested PCR*), o teste rápido para malária *Optimal-IT*, hemograma, enzimas hepáticas, avaliação da coagulação e da inflamação, além de sorologias para febre amarela, leptospirose, hepatites A, B, C e D, dengue, e HIV. Além disso, foram pesquisados traço falcêmico e outros polimorfismos relacionados à malária. Os fatores de exclusão foram, além da positividade nas sorologias pesquisadas (exceto para Hepatite B em um sub-estudo), a referência de alcoolismo crônico, câncer ou doença degenerativa e uso de imunossupressores. Todos os exames foram realizados no laboratório da Faculdade São Lucas-RO, LACEN-BA, Escola de Farmácia da UFBA e na FIOCRUZ-BA. Os indivíduos positivos para o plasmódio foram seguidos por 30 dias, quando novos exames foram realizados. Indivíduos infectados que permaneceram sem apresentar sintomas de malária neste período foram considerados portadores de malária assintomática. Aqueles que apresentaram sintomas de complicação, com hospitalização e/ou morte foram considerados graves. Após a

utilização dos critérios sorológicos de exclusão, os indivíduos foram categorizados de acordo com a apresentação clínica da malária em: não infectados (n=183) e os com malária assintomática (n=202), sintomática não complicada (n=195) e apresentando qualquer sintoma que possa ser classificado como malária grave de acordo com os critérios da Organização Mundial da Saúde (n=19). A partir deste ponto, as investigações variaram de acordo com o sub-estudo. No estudo do diagnóstico, várias técnicas diagnósticas foram comparadas, incluindo um software baseado em redes neurais artificiais. No segundo estudo, a sorologia anti-saliva do vetor *Anopheles darlingi* foi usada para estimar exposição ao *P. vivax* utilizando curvas ROC. No terceiro estudo, o impacto da hepatite B na apresentação clínica da malária foi testada. O quarto estudo faz uma descrição do perfil inflamatório e imunológico dos pacientes com malária vivax grave. O quinto estudo testa o estresse oxidativo como biomarcador da malária vivax grave, através da medida da superóxido dismutase-1 no plasma. Por fim, o sexto trabalho traz experimentos *in vitro* para explicar a relação entre a malária, hemólise e a desregulação imunológica.

5. MANUSCRITOS

5.1 MANUSCRITO I

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

Este trabalho compara a eficácia de diferentes testes diagnósticos na identificação de malária sintomática e assintomática. Além disso, um programa computacional utilizando redes neurais artificiais foi desenvolvido para tentar diagnosticar ativamente casos de malária assintomática.

Resumo dos resultados: O método molecular mostrou a mais alta performance para o diagnóstico da malária. O teste rápido foi superior à microscopia nos casos de baixa parasitemia, mas apresentou baixa performance no diagnóstico de infecções mistas. A microscopia apresentou apenas 61,25% de diagnósticos corretos casos assintomáticos. O sistema MalDANN usando apenas dados epidemiológicos apresentou-se pior do que a microscopia (56% de acertos). Entretanto, ao acrescentar dados de citocinas plasmáticas (IL10 e IFNgama), a performance do software aumentou sensivelmente (80% de acertos).

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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

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Abstract

Background: Accurate malaria diagnosis is mandatory for the treatment and management of severe cases. Moreover, individuals with asymptomatic malaria are not usually screened by health care facilities, which further complicates disease control efforts. The present study compared the performances of a malaria rapid diagnosis test (RDT), the thick blood smear method and nested PCR for the diagnosis of symptomatic malaria in the Brazilian Amazon. In addition, an innovative computational approach was tested for the diagnosis of asymptomatic malaria.

Methods: The study was divided in two parts. For the first part, passive case detection was performed in 311 individuals with malaria-related symptoms from a recently urbanized community in the Brazilian Amazon. A cross-sectional investigation compared the diagnostic performance of the RDT Optimal-IT, nested PCR and light microscopy. The second part of the study involved active case detection of asymptomatic malaria in 380 individuals from riverine communities in Rondônia, Brazil. The performances of microscopy, nested PCR and an expert computational system based on artificial neural networks (MalDANN) using epidemiological data were compared.

Results: Nested PCR was shown to be the gold standard for diagnosis of both symptomatic and asymptomatic malaria because it detected the major number of cases and presented the maximum specificity. Surprisingly, the RDT was superior to microscopy in the diagnosis of cases with low parasitaemia. Nevertheless, RDT could not discriminate the *Plasmodium* species in 12 cases of mixed infections (*Plasmodium vivax* + *Plasmodium falciparum*). Moreover, the microscopy presented low performance in the detection of asymptomatic cases (61.25% of correct diagnoses). The MalDANN system using epidemiological data was worse that the light microscopy (56% of correct diagnoses). However, when information regarding plasma levels of interleukin-10 and interferon-gamma were inputted, the MalDANN performance sensibly increased (80% correct diagnoses).

Conclusions: An RDT for malaria diagnosis may find a promising use in the Brazilian Amazon integrating a rational diagnostic approach. Despite the low performance of the MalDANN test using solely epidemiological data, an approach based on neural networks may be feasible in cases where simpler methods for discriminating individuals below and above threshold cytokine levels are available.

Background

Despite global efforts, the malaria burden is increasing worldwide, with almost two million estimated deaths annually [1]. The lack of precise malaria diagnosis

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remains an important obstacle to the treatment adherence and effectiveness and clinical management of severe cases. Additionally, the invasiveness and expense of tests limit their utilization in asymptomatic individuals.

Within the Brazilian Amazon, the microscopic detection and identification of *Plasmodium spp.* in Giemsa-stained blood smears from individuals presenting with malaria-like symptoms persists as the gold standard for the diagnosis of malaria and is mandatory to obtain access to the anti-parasitic treatment. Microscopic parasitological diagnosis requires continued personnel training and supervision of users in addition to a minimum laboratory structure, which is difficult to maintain in remote areas of the rainforest. Additionally, such a test is prone to large observer-related variation [2,3] and lacks sensitivity when performed by non-expert laboratory microscopists [4].

Other diagnostic methodologies have arisen to overcome the inefficient malaria diagnosis, such as PCR-based genetic tests. Nested PCR and real time PCR present higher sensitivity and specificity to malaria diagnosis compared to light microscopy [5,6]. Nevertheless, these molecular assays are costly and require even more laboratory support and personnel than microscopy, making it difficult to use routinely in the endemic areas. Rapid immunochromatographic tests (rapid diagnostic test, RDT) do not require laboratory support, are easily read and can reach a sensitivity similar to that commonly achieved by well-performed microscopy [7]. Nevertheless, most field evaluations of malaria RDTs were performed in countries with very high malaria endemicity [8,9], and validation studies in the Amazonian region are still scarce.

Within the Rondônia State in the Brazilian Amazon, the incidence of malaria and the occurrence of drug resistant cases are increasing [10]. In Buritis, a recently urbanized municipality, this situation is worsened by the lack of infrastructure of the health care system and the malaria control program. In addition, many other infectious diseases with similar clinical presentations, such as yellow fever, dengue and leptospirosis, are also common in this area, and the correct malaria diagnosis is of utmost importance to the adequate management of the patients. Certainly, one of the determining factors for morbidity and mortality is the delayed access to the health care. Moreover, the incidence of asymptomatic *Plasmodium* infection is very high in the Brazilian Amazon [11], further compounding the problem of malaria diagnosis. These individuals are not screened by the health care system, but they can transmit *Plasmodium* to uninfected *Anopheles* mosquitoes [12] and may represent important reservoirs. Therefore, the development of simple and noninvasive diagnostic tools is critical to hamper the spread of this infection.

Herein, the diagnostic effectiveness between an RDT (Optimal-IT), field microscopy and nested PCR was compared in individuals with malaria-related symptoms from an Amazonian region, which presents an increasing incidence of malaria [10]. Furthermore, a computational expert system based on artificial neural networks using epidemiological and clinical information was developed in an attempt to diagnose asymptomatic *Plasmodium* infection, and it was compared to field microscopy and nested PCR.

Methods

Ethics

This study was approved by the Ethical Committee of the São Lucas University, Rondônia, Brazil, for the human subject protocol. The clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. All participants or legal guardians gave written informed consent before patients entered the study.

Participants and sampling

This study was performed in Rondônia State in the southwestern Brazilian Amazon. Within this region, the malaria transmission is unstable, with an increasing number of cases being detected annually from April to September [13]. Most malaria cases are caused by *P. vivax*. The prevalence of *P. falciparum* infection in the Brazilian Amazon is 23.7% [10], and the case detection of *Plasmodium malariae* is about 10% in Rondônia [14].

For the first part of the study, a cross-sectional investigation was performed between May 2006 and September 2007 in Buritis, Rondônia, Brazil (10°12'43" S; 63°49'44" W), a recently urbanized municipality with high prevalence of symptomatic malaria [10]. Passive malaria case detections were carried out in individuals who sought care at the diagnostic centers of the Brazilian National Foundation of Health (FUNASA), responsible for malaria control in the country. The purpose of this sampling method was to identify individuals with malaria presumptive symptoms. A total of 311 subjects enrolled in this part of the study.

To test the efficacy regarding the diagnosis of asymptomatic *Plasmodium* infection, riverine communities close to Demarcação, Rondônia, Brazil (8°10'04.12" S; 62°46'52.33" W), in which a high prevalence of asymptomatic *Plasmodium* infection has been reported [11], were studied. Active case detection was performed, which included home visits with interviews, clinical evaluations, and blood collection for nested PCR and cytokine measurements. Participants without any clinical evidence of malaria infection were assessed. All individuals who were living in the endemic area for more than six months and were asymptomatic were invited to be ini-

tially included in the study. Hence, a total of 380 individuals enrolled in the second part of the study. The baseline characteristics of the participants are illustrated in Table 1.

The malaria diagnosis

The individuals were examined and interviewed by a trained physician. In the first part of the study, the thick blood smear and the Optimal-IT RDT (DiaMed China Ltd, Hong Kong, China) were run at the same time. The Optimal RDT was performed according to the manufacturer's instructions. For estimation of parasitaemia, experienced malaria field microscopists from the FUNASA malaria diagnostic center counted parasitaemia on slides using the thick film method. All the slides were stained using Giemsa pH 7.2. The results were reported as parasites/μL. In addition, 300 μL of total blood were collected in EDTA-treated tubes and stored for the nested PCR. The molecular diagnosis of malaria infection was performed in all subjects using the nested PCR technique described previously [15]. To control for cross-contamination, one uninfected blood sample was included for every twelve samples processed. Fifteen percent of positive PCR samples were re-tested to confirm the amplification of plasmodial DNA. Part of the molecular assays was performed in the field laboratory facility (USP/ICB5, Monte Negro, Rondônia, Brazil). All tests were repeated and confirmed at the main laboratory at the Centro de Pesquisas Gonçalo Moniz, Bahia, Brazil. To certify that the individuals with a positive nested PCR test really had symptomless *Plasmodium* infections, they were followed for 30 days. Only the individuals who remained without malaria-related symptoms and positive nested PCR test after this period were classified as asymptomatic malaria cases.

Expert System Based on Artificial Neural Networks

To identify asymptomatic *Plasmodium* infection, an expert system based on Artificial Neural Networks (ANN) [16] was developed using the epidemiological and clinical data. The software, called MalDANN (Malaria Diagnosis by Artificial Neural Networks), was built and validated using the data made available by a recent survey performed in malaria endemic areas in Rondônia State, Brazil, during 2006-2007, which was intended to study more deeply the causes that lead to asymptomatic malaria (unpublished observations). The MalDANN was developed in MATLAB 7.1 (MathWorks, Natik, MA, USA) using the Neural Network Toolbox for the construction of ANN.

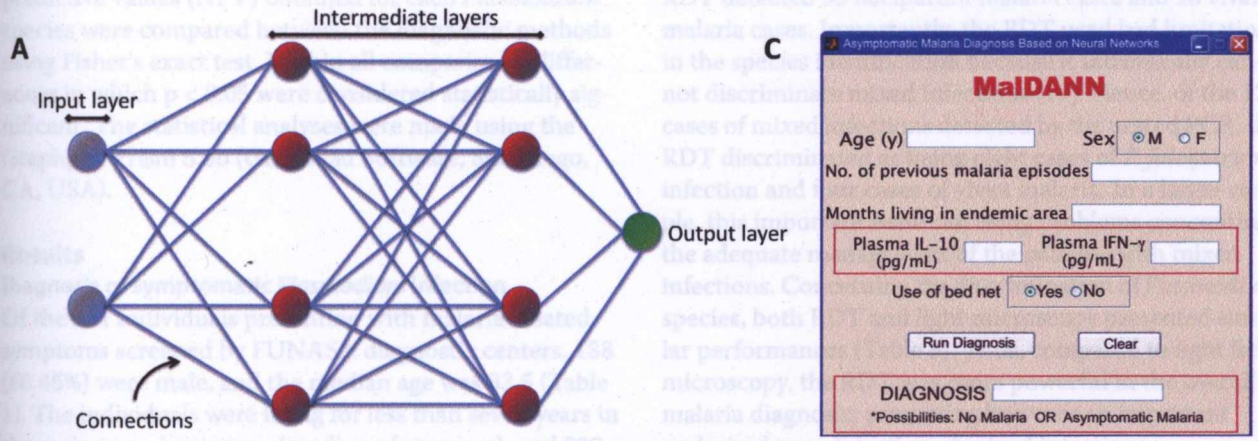
The database provided by the survey contained 380 records with information from non-infected individuals (n = 178) and those with asymptomatic malaria (n = 202) (infected with *P. vivax* and/or *P. falciparum*) according to the nested PCR and clinical evaluation described above. The objective was to develop a helpful method for discriminating asymptomatic plasmodial infections from uninfected cases.

The artificial neural network used in MalDANN was the Multilayer Perceptron because it is indicated for use in pattern recognition and provides the solution of problems not linearly separable [16,17] (Figure 1A). The network had one input layer (with seven neurons), two hidden layers (intermediate layers with four neurons each), one for each feature of the patient, and an output layer with only one neuron responsible for generating the diagnosis. The choice of activation functions of the layers of the neural network was made after a simulation of the activation functions provided by MATLAB. The best results were yielded by the function *tansig* in the input layer and hidden layers and the function *purelin* in the output layer. The network was trained using the back propagation technique in the Levenberg-Marquardt algorithm because it is very efficient when dealing with networks that have no more than a few hundreds of connections to be adjusted [18].

From the 380 records available in the database, a group of 300 records, approximately 80% of the total, were used for training, leaving 80 records for validation, approximately 20% of the total. In order to prevent a dominant class, a

Table 1: Baseline characteristics of the subjects.

	Passive case detection	Active case detection
Number of participants	311	380
Age - years - median (range)	33.5 (4-65)	29.6 (10-72)
Male	188 (60.45%)	245 (64.47%)
Time of residence in the area - years - median (range)	6 (0.5-25)	14 (0.530)
Number of patients who reported previous malaria infections	303 (97.43%)	368 (96.84%)
Number of previous malaria infections reported - mean (range)	5 (0-12)	13.5 (9-45)



B **General design of the Artificial Neural Network used by the MalDANN software**

Software version	Input layer		02 intermediate layers	Output layer
	Neurons	Variables inputted	Neurons	Neurons
MalDANN Epidemiologic	31 neurons	Epidemiological variables Age Sex Previous malaria episodes Months living in the endemic area Use of bed net	10 neurons	01 neuron
	Epidemiological variables*		Data processing to identify patterns	Generation of malaria diagnosis
MalDANN Epidemiologic + cytokines	07 neurons	Cytokine plasma levels (pg/mL) IL-10 IFN-γ	10 neurons	01 neuron
	Epidemiological variables + cytokine levels		Data processing to identify patterns	Generation of malaria diagnosis

Figure 1 General design of the Artificial Neural Network used by the MalDANN software. (A) The neural network used by the MalDANN software was based on the Multilayer Perceptron, which consists of: (i) one input layer, where the standards and data are presentedto the neural network; (ii) intermediate (or hidden) layers, where all the processing of the neural network is performed; and (iii) one output layer, in which the result of the network is presented to the observer. (B) Two software versions were created using different neural network structures to perform the diagnosis of asymptomatic *Plasmodium* infections. One version used epidemiological variables, and plasma levels of IL-10 and IFN-gamma were added to the epidemiological variables in the second version. (C) The intuitive interface of the MalDANN software was developed in order to facilitate the input of the data into the artificial network. * First, 31 epidemiological variables were added to the system for data mining. Of these, five variables presented very strong association with the asymptomatic malaria. The same five variables were added to the MalDANN version that used cytokine data.

fact that could affect the training and evaluation of results, both the training data and the validation data had a balanced proportion of non-infected and asymptomatic infections.

Plasma cytokine measurements

During the active search for subjects with asymptomatic *Plasmodium* infection, plasma levels of interleukin-10 (IL-10) and interferon-gamma (IFN-gamma) were measured using the Cytometric Bead Array - CBA® (BD Biosciences Pharmingen, USA) according to the manufacturer's protocol, with all samples run in a single assay in the main laboratory at the Centro de Pesquisas Gonçalo Moniz, Bahia, Brazil. The flow cytometric assay was performed and analyzed by a single operator, and standard curves were derived from cytokine standards. The cytokine levels were used for other studies addressing pathogenic aspects of malaria in this region [15], and the information was used in the present study to check the impact of the cytokine balance on the prediction of asymptomatic malaria by the MalDANN software. Further, Receiver operator characteristic (ROC) curves were created with the values of each cytokine, and cut-off values presenting higher sensitivity and specificity were chosen to discriminate asymptomatic *Plasmodium* infections. The MalDANN software used this additional information together with clinical and epidemiological data to enhance the power of prediction of asymptomatic malaria cases.

Statistical analysis

The overall performances of diagnostic methods were compared using Fisher's exact test (when two methods were compared) or a chi-square test (when three methods were compared). The results for the sensitivity, specificity, positive predictive values (PPV), and negative

predictive values (NPV) obtained for each *Plasmodium* species were compared between the diagnostic methods using Fisher's exact test. Within all comparisons, differences in which $p < 0.05$ were considered statistically significant. The statistical analyses were made using the Graphpad Prism 5.0b (GraphPad Software, San Diego, CA, USA).

Results

Diagnosis of symptomatic *Plasmodium* infection

Of the 311 individuals presenting with malaria-related symptoms screened by FUNASA diagnostic centers, 188 (60.45%) were male, and the median age was 33.5 (Table 1). The individuals were living for less than seven years in the malaria endemic area (median of six years), and 303 (97.43%) of them reported at least one previous symptomatic *Plasmodium* infection (median number of previous malaria episodes: 5; interquartile interval: 0-12).
Concerning the overall performance for the malaria diagnosis independently of the parasite species, the nested PCR resulted in the highest number of positive exams (173, 55.63% of the individuals), significantly superior to the RDT (154, 49.52%; $p = 0.022$, Fisher's exact test) and the field microscopy (141, 45.34%; $p = 0.013$, Fisher's exact test). As expected, the general positivity of the RDT was equivalent to the microscopy ($p = 0.81$; Fisher's exact test). Given that the nested PCR test presented higher positivity, it was considered as the gold standard to calculate the power of the two other tests. Therefore, for the diagnosis of symptomatic *Plasmodium sp.* infection, the RDT presented a sensitivity of 89.02% (95% CI: 83.38%-93.26%), a specificity of 100% (95% CI: 97.36%-100%), a positive predictive value (PPV) of 100% (95% CI: 97.36%-100%) and a negative predictive value (NPV) of 87.90% (95% CI: 81.75%-92.55%). Surprisingly, the light microscopy presented a lower sensitivity (81.50%; 95% CI: 74.9%-87.0%), an equivalent specificity (100%; 95% CI: 97.36%-100%) and PPV (100%; 95% CI: 97.42%-100%), and a lower NPV (81.18%; 95% CI: 74.48%-86.75%) than the RDT. Within the subjects evaluated in this study, no *P. malariae* case was detected. Under this circumstance, we decided to consider *P. non-falciparum* infection as *P. vivax* malaria cases for the RDT results.
Furthermore, the concordance of diagnosis in regard to the identification of the *Plasmodium* species was assessed (Table 2). The nested PCR detected a total of 107 individuals infected solely with *P. vivax* (61.84% of the positive cases), 53 individuals infected solely with *P. falciparum* (30.63% of the positive cases) and 13 cases of mixed infection (*P. vivax* + *P. falciparum*; 7.51% of the positive cases). The light microscopy detected 84 cases of vivax malaria (23 cases fewer than nested PCR), 45 cases of falciparum malaria (eight cases fewer than nested PCR), and 12 mixed malaria cases (one fewer than nested PCR). The RDT detected 56 falciparum malaria cases and 98 vivax malaria cases. Importantly, the RDT used had limitations in the species identification because it intrinsically cannot discriminate mixed infections [19]. Hence, of the 12 cases of mixed infections detected by the nested PCR, the RDT discriminated as being eight cases of *P. falciparum* infection and four cases of vivax malaria. In a larger sample, this important issue can bring problems concerning the adequate management of the patients with mixed infections. Concerning the discrimination of *Plasmodium* species, both RDT and light microscopy presented similar performances (Table 3). Thus, compared to light field microscopy, the RDT was more powerful in the overall malaria diagnosis, presenting however an important undesired non-detection of mixed infections.
The Table 4 presents the concordance of the tests' results according to the *Plasmodium* parasitaemia determined by light microscopy. Interestingly, a total of 32 patients were considered negative for malaria by the field microscopy specialized technicians from the FUNASA centers and were positive by nested PCR (23 vivax and nine falciparum malaria cases), while the RDT detected 13 cases (10 *P. vivax* and three *P. falciparum* infections). It was then possible that these patients had a parasitaemia below 100 parasites/μL because they were positive in two qualitative tests and negative by the parasite quantification using microscopy. On the other hand, the concordance of the results among the tests was similar when the patients presented with higher parasitaemia, except for the known absence of mixed infections detected by the RDT (Table 4). Further, the performance of RDT with microscopy in the infected subjects presenting with low parasitaemia, which was defined as <500 parasites/μL, was compared. The RDT was superior to microscopy concerning the diagnosis of *Plasmodium sp.* (76% vs. 59%, respectively), *P. falciparum* (75% vs. 63%, respectively) and *P. vivax* (76% vs. 58%, respectively) infections, with similar specificities and PPV (Table 5). Nevertheless, the microscopy had a higher NPV for *P. vivax* infections (88% vs. 75%, respectively). These findings indicate that for this endemic area, the RDT is superior to field light microscopy to identify individuals with low parasitaemia, albeit not detecting a few cases of mixed infections.

Diagnosis of asymptomatic *Plasmodium* infection

The next step was to assess the diagnosis of symptomless *Plasmodium*-infected individuals who are common in the Amazonian riverine communities [11] and may serve as infection source in endemic areas [12]. Previous studies have shown that individuals with asymptomatic malaria display distinct epidemiological characteristics from the symptomatic malaria cases [11]. In the present study, assessing another group of subjects from a riverine community, the field microscopy test correctly diagnosed

Table 2: Identification of symptomatic malaria cases: comparison among the field light microscopy, the Optimal-IT RDT and the nested PCR.

Microscopy	Optimal-IT®			Nested PCR			
	Pf	Pnf	Negative	Pf	Pv	Pf + Pv	Negative
Negative	3	10	157	9	23	0	138
P. falciparum	45	0	0	44	0	1	0
P. vivax	0	84	0	0	84	0	0
P. vivax + *P. falciparum*	8	4	0	0	0	12	0
Total	56	98	157	53	107	13	138

Pf = *Plasmodium* falciparum, Pv = *Plasmodium* vivax, Pnf = Non falciparum *Plasmodium*.

only 61.25% of the samples (sensitivity: 22.5%; specificity: 100%; Figure 2A) when the nested PCR was considered the gold standard.

One artificial neural networks-based test (MalDANN) was developed, and fed with a data bank built during another study (Andrade *et al.* unpublished data). Initially, the information provided by the data bank included test results of several immunological parameters, socioeconomic, environmental, clinical and epidemiological data (Figure 1A-C). A multivariate analysis of such a databank was used to evaluate whether the variables of gender, age, number of previous malaria episodes, time of residence in the endemic area and the use of bed nets were associated with the asymptomatic cases (Figure 1B). In this analysis, all variables except for gender and the use of a bed net were associated to some extent with asymptomatic infection (Andrade *et al.* unpublished data). The ANN pre-processing of the same data displayed better prediction results when all of the cited variables were used, including gender and the use of a bed net.

These variables were then selected to be inputted in the MalDANN for the validation process in the present study. With this initial configuration, it was observed that the network did not reach an acceptable error rate (data not shown). It was noticed that among the training data were data from patients with very similar values but with different diagnoses. Hence, there was a need for greater number of neurons to process and better differentiate the pattern of input data. A new network was created using ten neurons in the hidden layers, keeping the other settings (Figure 1B). Thus the network achieved the error rate of 10-13 in 900 times, an error level considered acceptable for the proposed problem. After the software was designed, a very intuitive interface was developed to validate the ANN (Figure 1C).

Further, the diagnostic performances of the MalDANN and light microscopy were compared, considering nested

Table 3: Performance of light microscopy and Optimal-IT in the discrimination of *Plasmodium* species.

Plasmodium sp.	Diagnostic test	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
P. vivax	Microscopy	80% (71.7-86.7)	100% (98.1-100)	100% (96.2-100)	88.8% (83.8-92.7)
	Optimal-IT	81.7% (73.6-88.1)	100% (98.1-100)	100% (96.3-100)	89.7% (84.8-93.4)
P. falciparum	Microscopy	86.4% (75.7-93.6)	100% (98.5-100)	100% (93.7-100)	96.5% (93.4-98.4)
	Optimal-IT	84.8% (73.9-92.5)	100% (98.5-100)	100% (93.6-100)	96.1% (92.9-98.1)

The overall performance of Optimal-IT and light microscopy were compared to the nested PCR as the gold standard. No significant statistical difference was found between the tests for the diagnosis of both *P. vivax* and *P. falciparum*. CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Table 4: Identification of symptomatic *Plasmodium* infection cases according to the parasitaemia.

Parasites/μL	Total	Microscopy				Optimal-IT*			Nested PCR			
		Pf	Pv	Pf+Pv	Negative	Pf	Pnf	Negative	Pf	Pv	Pf+Pv	Negative
Not detected	138	0	0	0	138	0	0	138	0	0	0	138
<100*	32	0	0	0	32	3	10	19	9	23	0	0
100 - 500	47	1	32	0	0	1	32	0	1	32	0	0
		5				5			5			
501 - 5,000	24	1	0	5	0	2	2	0	1	0	6	0
		9				2			8			
5,001 - 50,000	56	1	40	5	0	1	42	0	1	40	5	0
		1				4			1			
>50,000	14	0	12	2	0	2	12	0	0	12	2	0
Total	311	4	84	12	170	5	98	157	5	107	13	138
		5				6			3			

Pf = *Plasmodium* falciparum, Pv = *Plasmodium* vivax, Pnf = Non-falciparum *Plasmodium*.

* 32 subjects were negative for *Plasmodium* infection by field light microscopy. Nevertheless, nested PCR attested positive results (Optimal_IT identified 13 of them). Thus, these individuals probably present very low parasitaemia.

PCR as the gold standard. Using exclusively the epidemiological data, the software diagnosed correctly only 56% of the samples (sensitivity: 70%; specificity: 28%), exhibiting lower performance than microscopic diagnosis due to increased number of false positive exams (72% vs. 0% of the samples tested, respectively; Figures 2A and 2B). Additionally, the software was not able to discriminate the *Plasmodium* species. These data indicate that non-epidemiological variables may determine the occurrence of asymptomatic *Plasmodium* infection. To address whether the cytokine balance had a determining role in this process, plasma levels of IL-10 and IFN-gamma were measured, and the results were added into the neural network of the software. Strikingly, the software updated with the cytokine data correctly diagnosed asymptomatic malaria in 80% of the samples (sensitivity: 67.5%; specificity: 92.5%; Figure 2C) with a performance above field microscopy. Noteworthy, using the cytokine information (Figure 1B-C), the neural network reduced the false negative cases by 45% (Table 6). Interestingly, when added to the MalDANN system, other routine plasmatic biochemical laboratory exams, such as C reactive protein, fibrinogen, creatinine, haemoglobin, total bilirubin, direct bilirubin and indirect bilirubin, did not improved the diagnostic performance compared with the microscopy (MalDANN 60.4% vs. microscopy 61.25%; p = 0.6).

Discussion

The present study adds some relevant issues for the diagnosis of malaria in the Amazonian region. Firstly, only 55.63% of the individuals who sought care in a malaria diagnosis center presenting with malaria-related symptoms were diagnosed by the most sensitive diagnostic method applied in this study. The individuals with other diseases looked for exclusion of malaria before seeking care in a regular health clinic, possibly due to the high prevalence of malaria in the municipality. This custom can lead to a delay of the correct diagnosis and in severe cases, could compromise an adequate early management, directly impacting the prognosis and the cost of the health care.

The use of nested PCR as the gold standard was done when it was noted that it presented the highest sensitivity. Considering the principle of this molecular assay, in which small fragments of *Plasmodium* DNA can be detected, the results were not surprising. Nevertheless, it is worthy to evidence that until today the nested PCR is available only as a research tool, and the cost and technical complexity of this technique hamper the its use in quotidian screening and survey works. The rationale for choosing PCR as gold standard was the necessity of testing the diagnosis accuracy of the field microscopy and the RDT. In this study, 10.3% of the symptomatic individuals with a positive nested PCR (nine *P. falciparum* and 23 *P. vivax* cases) were negative by light field microscopy. Although these individuals probably had low parasitaemia, they were symptomatic and did not receive antimalarial treatment because of the negative microscopy exam. This finding reinforces that the field microscopists from this area need continued refinement, and invest-

Table 5: Overall performance of microscopy and Optimal-IT in the discrimination of symptomatic malaria cases presenting with low parasitaemia.

Diagnostic method	*Plasmodium* species	Sensitivity	Specificity	PPV	NPV
Optimal-IT®	*Plasmodium non falciparum*	76%*	100%	100%	75%
	P. falciparum	75%†	100%	100%	97%
	Plasmodium sp	76%*	100%	100%	88%
Microscopy	*P. vivax*	58%	100%	100%	88%†
	P. falciparum	63%	100%	100%	96%
	P. vivax + *P. falciparum*	59%	100%	100%	81%

Values represent data from patients with <500 parasites/μL of blood determined by light microscopy. Nested PCR was considered the gold standard. PPV, positive predictive value; NPV, negative predictive value. The results for the sensitivity, specificity, PPV and NPV obtained for each *Plasmodium* species were compared between the diagnostic methods using Fisher's exact test. *p < 0.01; †p < 0.05.

ments are necessary to improve the quality of the malaria screening. It also indicates that the large demand for microscopic tests in this area, including those from patients with other infections, could contribute to the reduced quality of the tests. Recent evidence indicates that there is a large inter-rater reliability of the parasite counts for the malaria diagnosis [3]. The thin film method is not feasible at a parasitaemia below 500 parasites per microlitre, while the thick film method gives slightly better inter- rater agreements [3]. Moreover, it is well known that most routine malaria microscopists require constant retraining, and that their ability to detect a high proportion of malaria cases is suspect [20].

In addition, the RDT presented higher effectiveness in the identification of malaria cases with low parasitaemia than the light microscopic test. Many other studies worldwide have indicated diverse findings [5,21,22]. This result reinforces the idea that assays for rapid diagnosis have the potential to enhance diagnostic capabilities in those instances in which skilled microscopy is not readily available [23]. In order to identify individuals with low parasitaemia neglected by the light microscopy screening in this endemic area, the use of a RDT is advisable in symptomatic individuals who presented a negative thick blood smear exam. This method should be tested in field conditions but it will likely expand the detection of infected individuals and may favour the early clinical

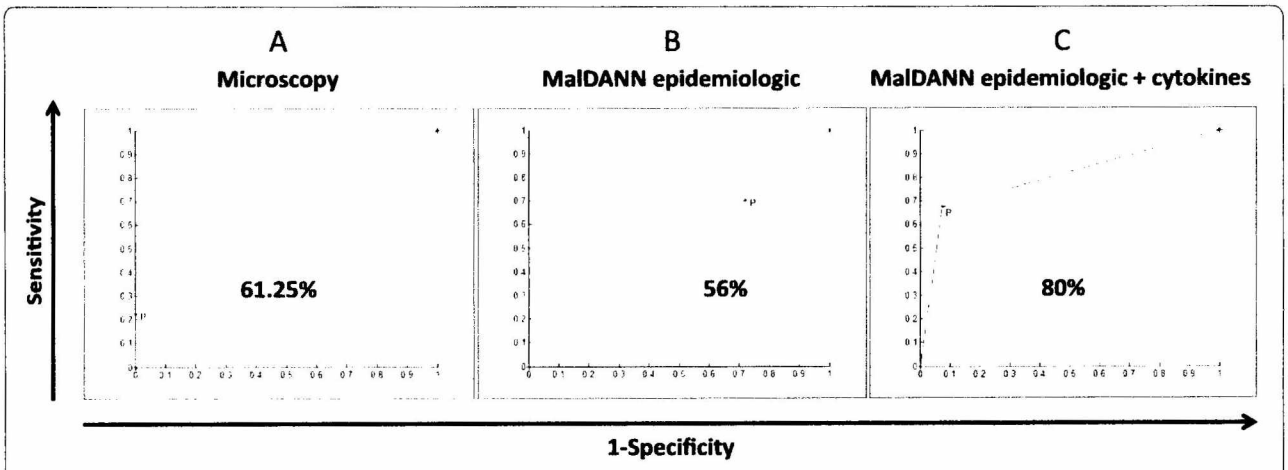


Figure 2 Performance in discriminating asymptomatic *Plasmodium* infections. In this investigation in an Amazonian riverine community, 380 apparently healthy individuals exhibiting no malaria-related symptoms were screened for *Plasmodium* infection by light field microscopy. Before the blood collection, the individuals were interviewed, and epidemiological data was obtained according to the methods. Whole blood and plasma samples were stored for nested PCR and cytokine measurements. The software MalDANN used epidemiological data only or in combination with the plasma levels of IFNgamma - and IL-10 to estimate the discrimination of the asymptomatic malaria cases. The overall performances of the field light microscopy (A) and the MalDANN (B and C) were compared using ROC curves, considering the nested PCR as the gold standard. The X-axes represent 1- specificity; the Y- axes represent sensitivity. Numbers inside the areas under the curves represent the percentages of correct diagnosis, which were statistically different using the chisquare-test (p = 0.002).

Table 6: Overall performance to discriminate asymptomatic malaria cases

Diagnostic method	Correct diagnosis (%)§**	True positive (%)**	True negative (%)***	False positive (%)***	False negative (%)**
Microscopy	61.25	22.5	100	0	77.5
MalDANN Epidemiologic	56	70	28	72	30
MalDANN Epidemiologic + cytokines	80	67.5	92.5	7.5	32.5

MalDANN: Malaria Diagnosis by Artificial Neural Networks. This diagnostic software was trained on the sample of 300 individuals actively screened for asymptomatic malaria and was validated in 80 other individuals, according to the methods. The diagnostic methods presented significantly different results estimated using the Chi-square test. §correct diagnosis involves both negative and positive correct exams. **$p < 0.01$; ***$p < 0.0001$.

intervention and adequate case management. In the sample of 311 individuals, this approach would have resulted in the use of 32 RDT tests, with a minor impact of the health care cost compared to the possible outcomes resulting from a delayed diagnosis.

Optimal-IT, the RDT used in the present study, cannot discriminate mixed infections. The discrimination between *P. vivax* and *P. falciparum* infections is critical because the drug therapies and the treatment durations are different. Actually, there are other RDT that can discriminate mixed infections [24], and these should be validated in this endemic area because the occurrence of mixed infections found here was 4.2%. The choice of using the Optimal-IT in this study was made because FUNASA was validating its use in the field during the study period. This work took advantage of this occasion and decided to compare the power of this RDT with other diagnostic tools.

The asymptomatic *Plasmodium* infection is a major problem in many regions worldwide [25,26], including the Brazilian Amazon [11]. Symptomless individuals probably develop clinical immunity to *Plasmodium* parasites after repeated infections [27], which lead to modifications on the host physiology that minimize the intensity of the symptoms, maintaining a very low parasitaemia for long periods [28]. While under this occult infection, these individuals have no reason to seek care in the malaria diagnosis centers. On the other hand, the quotidian primary care activities do not include active detection of asymptomatic malaria. Consequently, these symptomless individuals remain parasitaemic and can serve as a parasite source for uninfected mosquitoes [12], which in turn favours the spread of the infection. Knowledge ofthe prevalence of asymptomatic malaria cases in certain regions could assist in the implementation of control strategies, which may include treatment of asymptomatic *Plasmodium*-infected individuals.

Asymptomatic individuals frequently refuse to give blood for tests, which hampers the detection of symptomless plasmodial infections. Additionally, the routinely used thick blood smear exam displays a low performance in individuals with low parasite burdens, as is the case in asymptomatic *Plasmodium* infection [29]. Herein, a pilot investigation was performed addressing whether a computational system could discriminate asymptomatic malaria cases. For this purpose, major epidemiological determinants of asymptomatic malaria, such as the age, time of residence in the endemic area, number of previous malaria infections, gender and use of bed nets, were used. The technique of ANN was chosen because it offers good robustness against noise and typically works very well when no previous knowledge is available in order to facilitate the classification [18]. Thus, the network can be trained to recognize the pattern of the disease to be diagnosed from the medical database used. With a very intuitive interface that could be used by primary care professionals in the endemic areas, the software used an expert system based on neural networks [16]. The disappointing results obtained by the exclusive use of epidemiological data indicate that other complex factors may be more influential for the development of the asymptomatic *Plasmodium* infection. This idea was confirmed when the software performance significantly improved after the addition of information regarding IL10 - and IFN-gamma plasma measurements. In addition, other routine biochemical laboratory exams did not improved the MalDANN performance. These cytokines were chosen in the light of evidences that individually or as ratios they are associated with the malaria severity [15,30]. Besides the known epidemiological factors, the genetic background and/or the common occurrence of co-infections within the population may play a fundamental role on the occurrence of asymptomatic malaria.

Conclusion

This study shows that the chosen RDT (Optimal-IT) performed superiorly in discriminating symptomatic malaria cases with low parasitaemia than field microscopy,

although it did not discriminate mixed infections. An RDT for malaria diagnosis may find a promising use in the Brazilian Amazon integrating a rational diagnostic approach. Despite the low performance of the MalDANN test using solely epidemiological data, an approach basedon neural networks may be feasible in cases where simpler methods for discriminating individuals below and above threshold cytokine levels are available.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Wrote the paper: BBA; Performed data analysis: BBA and ARF; Performed the field study and clinical examinations: BBA, SMSN and LMAC; Performed molecular experiments: LLN, KFF and BBA; Designed and validated the Expert Based System Networks for malaria diagnosis: AMB and AD; Participated in the design of the study and helped with the manuscript: LMAC, EC and AB; Coordinated the study and helped to draft the manuscript: MBN. All authors have read and approved the final version of the manuscript.

Authors' information

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5.2 MANUSCRITO II

Anti-*Anopheles darlingi* saliva antibodies as marker of *Plasmodium vivax* infection and clinical immunity in the Brazilian Amazon.

Este trabalho avalia a possibilidade do uso da mensuração de anticorpos contra o sonicado da saliva do principal vetor da malária no Brasil para estimar exposição ao *P. vivax* e também a sua relação com a ocorrência da malária assintomática.

Resumo dos resultados: Os indivíduos com malária vivax apresentaram maiores níveis séricos de anticorpos anti-saliva do *Anopheles darlingi* do que os indivíduos não infectados. Os níveis de anticorpos mostraram alta performance na diferenciação entre indivíduos com malária vivax assintomática e os não infectados. Indivíduos com malária assintomática apresentaram níveis mais altos de anti-saliva e mais baixos da razão de citocinas IFNgama/IL10 do que os indivíduos com sintomas. Houve uma correlação inversa entre os valores de anti-saliva e a razão de citocinas.

Este trabalho foi publicado no periódico internacional *Malaria Journal* (Fator de Impacto JCR 2009 = 3.00) e recebeu 1428 acessos on-line nos primeiros três meses de publicação.

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Anti-*Anopheles darlingi* saliva antibodies as marker of *Plasmodium vivax* infection and clinical immunity in the Brazilian Amazon

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Abstract

Background: Despite governmental and private efforts on providing malaria control, this disease continues to be a major health threat. Thus, innovative strategies are needed to reduce disease burden. The malaria vectors, through the injection of saliva into the host skin, play important role on disease transmission and may influence malaria morbidity. This study describes the humoral immune response against *Anopheles (An.) darlingi* saliva in volunteers from the Brazilian Amazon and addresses the association between levels of specific antibodies and clinical presentation of *Plasmodium (P.) vivax* infection.

Methods: Adult volunteers from communities in the Rondônia State, Brazil, were screened in order to assess the presence of *P. vivax* infection by light microscopy and nested PCR. Non-infected volunteers and individuals with symptomatic or symptomless infection were randomly selected and plasma collected. *An. darlingi* salivary gland sonicates (SGS) were prepared and used to measure anti-saliva antibody levels. Plasma interleukin (IL)-10 and interferon (IFN)-γ levels were also estimated and correlated to anti-SGS levels.

Results: Individuals infected with *P. vivax* presented higher levels of anti-SGS than non-infected individuals and antibody levels could discriminate infection. Furthermore, anti-saliva antibody measurement was also useful to distinguish asymptomatic infection from non-infection, with a high likelihood ratio. Interestingly, individuals with asymptomatic parasitaemia presented higher titers of anti-SGS and lower IFN-γ/IL-10 ratio than symptomatic ones. In *P. vivax*-infected asymptomatic individuals, the IFN-γ/IL-10 ratio was inversely correlated to anti-SGS titers, although not for while in symptomatic volunteers.

Conclusion: The estimation of anti-*An. darlingi* antibody levels can indicate the probable *P. vivax* infection status and also could serve as a marker of disease severity in this region of Brazilian Amazon.

Background

Malaria continues to be one of the most serious public health problems worldwide, exacting a huge impact on human wellbeing, mainly in tropical and subtropical countries. A better understanding of the interactions between the host, the vector and the parasite could be valuable to indicate future strategies. In endemic regions, residents are frequently bitten by both uninfected and infected mosquitoes. There is also a progressive acquisition of immunity, leading to a decreased number of malaria clinical attacks related to increasing age and time residing in the endemic area [1,2]. Within the Brazilian Amazon, and mainly in riverine communities, the prevalence of asymptomatic malaria infection seems to be four to five times greater than the symptomatic infection [3-5]. Malaria clinical immunity has already been described in both *Plasmodium (P.) falciparum* [6] and *Plasmodium (P.) vivax* [7] infections and it seems to be related to higher titers of anti-*Plasmodium* antibodies [8]. On the other hand, anti-parasite response might not be the unique determinant of the occurrence of symptomless malaria, as asymptomatic patients maintain parasitaemia at low levels in addition to controlling the clinical symptoms [9]. Such asymptomatic carriers have developed just enough immunity to protect them from malarial illness but not from malarial infection. Regardless these facts, the specific mechanisms that underlie the occurrence of clinical immunity against the *Plasmodium* are not well understood.

In this scenario, the anopheline vector could play significant role in malaria clinical severity. Mosquito bites can induce immediate, delayed, and systemic hypersensitivity reactions in hosts [10]. Moreover, pre-exposure to the vector saliva may create an inhospitable environment for the establishment of the parasites transmitted by these insects. Mice repeatedly exposed to bites from uninfected *Anopheles (An.) stephensi* increase a pro-inflammatory T helper 1 biased response that limits *P. yoelii* infection [11]. In humans it has been shown that *An. gambiae* saliva is immunogenic for travelers transiently exposed to bites in African endemic areas [12], with the development of specific IgG and IgM antibodies. Specific anti-*An. gambiae* saliva IgG antibodies were also detected in young children from a seasonal malaria transmission region in Senegal, and antibody levels were higher in patients who developed clinical malaria episodes, suggesting that the estimation of humoral response to *Anopheles* salivary antigens can serve as potential marker for the risk of malaria [13]. Moreover, anti-*An. dirus* salivary protein antibodies occur predominantly in patients with acute *P. falciparum* or *P. vivax* malaria, whereas people from non-malarious areas do not carry such antibodies [14]. Little is known about anti-saliva humoral responses in other endemic areas, such as Latin America. In addition, the host response against the most widespread malaria vector in America, *An. darlingi*, is poorly explored. The objective of the present work was to measure the anti-saliva IgG responses against *An. darlingi* mosquitoes in the Brazilian Amazon and to evaluate the association of antibody levels with different clinical presentations of *P. vivax* infections.

Methods

Study localities

A cross-sectional study investigating determinant factors for asymptomatic *P. vivax* malaria was performed during 2007 (June to August) in Buritis (10°12'43" S; 63°49'44" W), a recent urbanized municipality, and Demarcação (8°10'04.12" S; 62°46'52.33" W), a riverine community of the Rondônia State, in the south-western part of Brazilian Amazon. In general, Rondônia has a flat topography, with an average elevation of 300 m above sea level. The climate is tropical, with a long rainy season from January till May. It is argued that the environmental changes caused by deforestation have favored the main malaria vector in Brazil *An. darlingi* [15]. Within the regions studied here, the malaria transmission is unstable, with increased number of cases being detected annually between April to September, and the risk of infection is moderate to high [16], with an Annual Parasite Incidence of 77.5 per 1,000 inhabitants in 2005 [17]. In the Brazilian Amazon, *P. vivax* accounts for the majority of malaria cases, while *P. falciparum* infection prevalence is 23.7% [17]. In addition, infection with *P. malariae* achieves 10% in Rondônia [18].

Volunteers

Active and passive malaria case detections were performed in the two communities studied. A small laboratory with necessary facilities was built inside the main centers for malaria diagnosis in Buritis and Demarcação. These diagnostic centers are linked to the Brazilian National Foundation of Health (FUNASA), responsible for malaria control in the Brazilian Amazon. Active case detection was made by visiting residences in regions pointed by the local health authorities as major areas of disease transmission. The individuals were examined and interviewed by a trained physician, and blood samples were collected for serological experiments. The malaria diagnosis was performed using two methods. First, patients were screened by thick smear examination using field microscopy and the parasitaemia (parasites/μL) was calculated in positive cases. Further, nested PCR was performed in all whole blood samples to confirm the diagnosis (as described below). Two individuals presenting *P. malariae* infection and 16 persons infected with *P. falciparum* were identified and excluded from the study. Hence, all the volunteers selected were negative for *P. falciparum* and/or *P. malariae* infection by both microscopic examination and nested PCR. Other exclusion criteria were chronic alcoholism,

severe chronic degenerative disease as well as HIV, HBV and HCV infections. A total of 204 volunteers were used in the study. All the positive cases were followed up for 30 days for the evaluation of malaria symptoms. Individuals who were positive for *P. vivax* infection and remained without fever (axilary temperature >37.8°C) and/or chills, sweats, strong headaches, myalgia, nausea, vomiting, jaundice, asthenia, and arthralgia for 30 days were considered asymptomatic, while in the presence of any listed symptom they were classified as symptomatic. The volunteers were stratified in three different groups according to the *P. vivax* malaria diagnosis and the clinical spectrum of the disease. Thus, 80 people were non-infected, 50 had asymptomatic infection and 74 were symptomatic. The baseline characteristics of the volunteers are listed in the Table 1. Three volunteers from asymptomatic infection group presented negative light microscopy exam, but *P. vivax* DNA was amplified by nested PCR (Table 1). This study was a part of the project approved by the Ethical Committee of the São Lucas University, Rondônia, Brazil, for the human subject protocol and is in compliance with the Helsinki Declaration. All participants gave written informed consent before entering the study.

Molecular malaria diagnosis

The molecular diagnosis of malaria infection was performed using the nested PCR technique, based on the Snounou protocols, with minimal alterations [19,20]. The target was the 18S rRNA gene, and genus- and species-specific primers were used in the assay. Briefly, 300 μL of whole blood collected on EDTA was prepared for DNA extraction through the phenol-chloroform method followed by precipitation with sodium acetate and ethanol. The first PCR rDNA amplification was performed with *Plasmodium* genus-specific primers named PLU5 and PLU6. Positive samples yielded a 1,200-bp fragment, which served as template for the nested reaction. The nested PCR amplification was performed with species-specific primers for 30 cycles at annealing temperatures of 58°C for *P. falciparum* (Fal1 and Fal2 primers), and 65°C for *P. vivax* (Viv1 and Viv2 primers) or *P. malariae* (Mal1 and Mal2 primers). The fragments obtained for *P. vivax* were of 120 bp, whereas for *P. falciparum* and *P. malariae* were 205 bp and 144 bp, respectively. The oligonucleotide sequences of each primer used are listed in Table 2. The products were visualized in 2% agarose gel stained with ethidium bromide. To control for cross-contamination, one uninfected blood sample was included for every twelve samples processed. Fifteen percent of positive PCR samples were re-tested to confirm the amplification of plasmodial DNA. All the tests were performed and confirmed at the Centro de Pesquisas Gonçalo Moniz (FIOCRUZ-BA).

Salivary Gland Sonicate (SGS) preparation

Salivary glands from field captured adult female *An. darlingi* mosquitoes were dissected and transferred to 20 μL

Table 1: Baseline characteristics of the volunteers.

Variable	Non-infected (n = 80)	*Plasmodium vivax* current infection: Asymptomatic (n = 50)	*Plasmodium vivax* current infection: Symptomatic (n = 74)	P value
Age – years*	30 (23–44.5)	44.5 (34.5–51)	27.5 (21–37)	0.0341†
Malaria episodes referred*	13.5 (11–18)	17.5 (13–21)	7 (1–13)	0.0283†
Time residing in the area – years				0.0185‡
<2	25 (31.3%)	8 (16%)	31 (41.9%)	
3–10	12 (15%)	12 (24%)	16 (21.6%)	
>10	43 (53.7%)	30 (60%)	27 (36.5%)	
Parasitaemia – parasites/μL				< 0.0001‡
ND§	80 (100%)	3 (6%) §	0	
100–<500	0	44 (88%)	34 (45.9%)	
500–<5,000	0	3 (6%)	5 (6.8%)	
5,000–<50,000	0	0	30 (40.5%)	
>50,000	0	0	5 (6.8%)	
IgG anti-SGS – O.D.*	0.06 (0.04–0.09)	0.13 (0.08–0.26)	0.095 (0.07–0.14)	< 0.0001†
Plasma IL-10 – pg/mL*	12.6 (7.4–19.2)	64.5 (7.3–86.0)	23.4 (9.5–58.4)	NS†
Plasma IFN-γ – pg/mL*	14.2 (0–32.0)	44.0 (10.5–101.0)	75.5 (38.8–243.5)	NS†

* Values plotted represent media and range

† Ordinal variables were compared between groups Kruskal-Wallis test with Dunn's multiple comparison test.

‡ Categorized variables were compared using chi-squared test. P values obtained in each test are plotted.

§ND: Six patients, out of 50 were negative for malaria infection by light microscopy, but were positive for *P. vivax* infection by nested PCR.

NS: Non significant.

Table 2: Primers used in Nested PCR reactions.

Primer	Oligonucleotide Sequence 5'-3'	Base pairs
PLU5	CCTGTTGTTGCCTTAAACTTC	1,200
PLU6	TTAAAATTGTTGCAGTTAAAA	
Fal1	TTAAACTGGTTTGGGAAAACCAAATATATT	205
Fal2	ACACAATGAACTCAATCATGACTACCCGTC	
Viv1	CGCTTCTAGCTTAATCCACATAACTGATAC	120
Viv2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	
Mal1	ATAACATAGTTGTACGTTAAGAATAACCGC	144
Mal2	AAAATTCCCATGCATAAAAAATTATACAAA	

PLU: *Plasmodium sp*, Fal: *Plasmodium falciparum*, Viv: *Plasmodium vivax*, Mal: *Plasmodium malariae*.

of 10 mM HEPES pH 7.0, 0.15 mM NaCl in 1.5-mL polypropylene vials, usually in groups of 20 gland pairs. Salivary glands were kept at -70°C until needed, when they were disrupted by sonication using a Branson Sonifier 450 homogenizer (Branson, Danbury, CT). The homogenates were centrifuged at 10,000 × g for 4 min and the supernatants were used for the experiments. Protein concentrations were measured by the bicinchonic acid method (BCA, Pierce, Rockford, Illinois, USA). As the salivary glands used in this study were obtained from field captured mosquitoes, *Plasmodium* contamination needed to be checked by nested PCR. Briefly, it was performed the DNA extraction of a sample from the same SGS pool used in the serological experiments using the Qiagen Generation Capture Card Kit (Cat. No. 159982; Qiagen, Santa Clara, California, USA). Further, the nested PCR was performed as described above, in duplicate samples. There was no amplification of DNA in both duplicates (data not shown).

Anti-An. darlingi *saliva serology*

Volunteer's sera were collected and kept at -70°C. Serological tests of all samples were performed in a single experiment, with duplicate samples. ELISA was performed as described elsewhere [14]. Briefly, plates were coated with *An. darlingi* salivary homogenate (SGS) equivalent to 1.5 μg/mL in carbonate buffer overnight at 4°C, then washed with PBS/0.05% Tween and blocked with PBS/0.1% Tween plus 0.05% BSA. Sera were diluted 1:100 with PBS/0.05% Tween and incubated overnight at 4°C. After further washings, the wells were incubated with alkaline phosphatase-conjugated anti-human IgG (Sigma-Aldrich, St. Louis, MO) at a 1:5,000 dilution. Following another washing cycle, the color was developed with p-nitrophenylphosphate. The reactions were blocked with NaOH and read at 405 nm using Soft Max-Pro Software v5 (Molecular Devices Corporation, Sunnyvale, California, USA) ELISA reader. The optical density (OD) values plotted represent the means between each sample duplicate, adjusted for the values from the blank wells.

Plasma cytokine measurement

Interleukin (IL)-10 and interferon (IFN)-γ plasma levels were measured using de Cytometric Bead Array – CBA® (BD Biosciences Pharmingen, San Diego, California, USA) according to the manufacturer's protocol.

Statistical analysis

Data were analyzed using the GraphPad Prism 5.00® (GraphPad Software Inc.). For the ordinal variables (age, referred malaria episodes, IgG, IL-10 and IFN-γ serum levels), differences between groups were calculated using the non parametric Kruskal-Wallis test with Dunn's multiple comparison post test. Chi-square test was used to compare differences regarding categorized variables (Time residing in the area and parasitaemia). Mann-Whitney test was used to compare differences in IgG levels between non-infected individuals and those with symptomatic or asymptomatic *P. vivax* infection. This test was also used to estimate significance in IFN-γ/IL-10 ratios from volunteers with asymptomatic or symptomatic *P. vivax* infection. To evaluate the cut off value of IgG anti-SGS predicting malaria infection or asymptomatic infection, we performed Receiver-operator characteristic (ROC) curves, calculated the Area under curve (AUC), and then estimated the likelihood ratio for the discrimination between the conditions analyzed. Fine Lowess curves were plotted to evidence the trend of the data presented in correlation analyzes. Spearman test was used to verify the significance in the correlations between cytokine levels and anti-SGS levels. Differences were considered significant at $P < 0.05$.

Results and discussion

In an attempt to check if the measurement of anti-SGS antibody levels could be a suitable method to estimate natural exposure to *P. vivax*, anti-SGS values obtained from non-infected individuals were compared to those from either symptomatic or asymptomatic infected volunteers. As seen in Figure 1A, infected patients presented higher levels of specific antibodies against *An. darlingi* salivary antigens than non-infected individuals. The variabil-

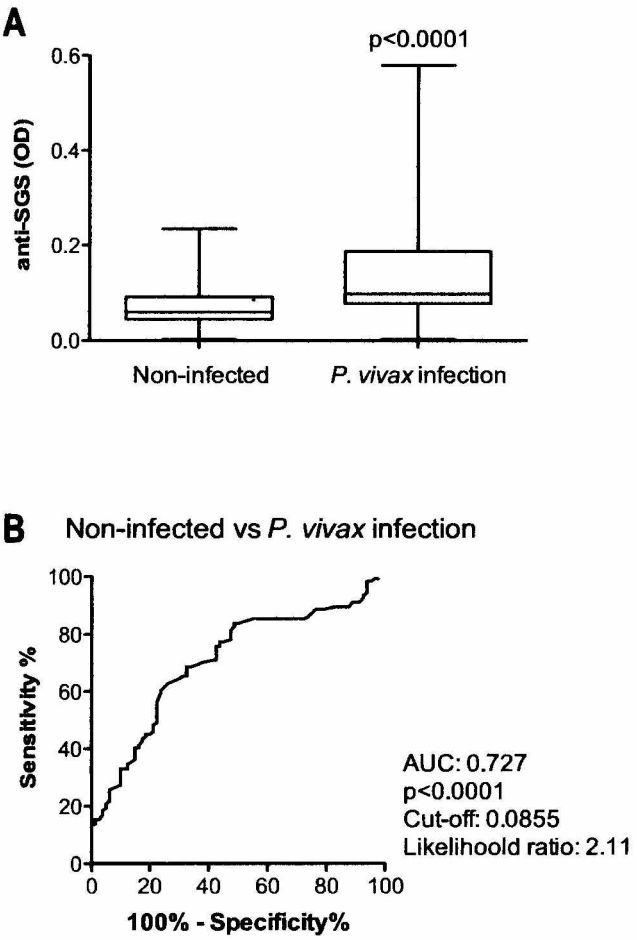

Figure 1

Anti-saliva IgG serum levels according to malaria occurrence. Sera were collected from non-infected individuals (n = 80), and from patients with *P. vivax* infection with or without symptoms (n = 124). An ELISA test was performed to assess the IgG anti-*An. darlingi* SGS. (A) Box plot graphs of IgG serum levels from non-infected individuals and from patients with *P. vivax* infection. Lines of the boxes represent 75th percentile, median and 25th percentile of the individual average OD values; whiskers represent the maximum and minimum values. Differences between groups were tested using Mann Whitney test. (B) ROC curve evaluating the threshold value of anti-SGS that separates non-infected individuals from *P. vivax* infection. Area under curve (AUC) calculated, together with the cut off value, which presents the higher likelihood ratio, and p values are plotted.

ity probably indicates individual differences in exposure to mosquito bites, even during the period of high malaria transmission, when these data were collected. Previous studies have also demonstrated a high variation in anti-*An. dirus* saliva antibody titers [14].

A ROC curve was built to assess the best anti-SGS OD value to discriminate *P. vivax* infection from the non-infected condition. A cut-off point of 0.0855 OD displayed a likelihood ratio to be infected of 2.11 indicating *P. vivax* infection (Figure 1B; AUC: 0.727; $p < 0.0001$). These data suggest that evaluation of anti-saliva antibodies could be a useful indicator to estimate exposure to *P. vivax* in this endemic area. High anti-SGS antibody levels were also proposed as putative biomarkers of exposure to bites of *An. stetephensi* or *An. gambiae* and also of risk of *P. falciparum* malaria [13]. In this study, besides suggesting exposure to bites, high anti-*An. darlingi* saliva antibody levels could also indicate exposure to *P. vivax*.

This work is the first to evaluate human immune response against salivary components of *An. darlingi*, the most widespread specie of *Anopheles* mosquitoes and the major malaria vector in the Americas [21]. In areas with unstable malaria transmission and moderate risk of infection, such as the Brazilian Amazon, adults, instead of children are largely affected by the disease. Hence, this study focused investigation on the adult population from a Brazilian endemic area.

Diagnosis of symptomatic malaria cases is routinely performed in the endemic areas. A real challenge for diagnosis is to discriminate asymptomatic *Plasmodium*-infected individuals from those with no malaria infection. Despite presenting no clinical manifestations, asymptomatic *Plasmodium*-infected individuals are able to transmit *Plasmodium* to uninfected mosquitoes [22]. Thus, asymptomatic persons could serve as important reservoirs, and the possibility of identifying them could be useful for malaria control. Asymptomatic individuals presented higher anti-SGS antibody levels than non-infected individuals (Figure 2A, $p < 0.0001$). A ROC curve to discriminate these two clinical conditions showed that a cut-off value of 0.0935 OD, with a likelihood ratio of 3.03, indicated asymptomatic infection (Figure 2B; AUC: 0.798; $p = 0.0001$). Considering solely the *P. vivax* infected patients, asymptomatic individuals presented higher levels of anti-SGS than symptomatic ones (Figure 3, $p = 0.0009$). Evaluation of antibodies against *An. darlingi* saliva may serve as a marker of *P. vivax* asymptomatic infection in this Brazilian malaria endemic area.

In order to explore immunopathological patterns in *P. vivax* infection, the correlation between the anti-SGS antibody levels and the serological cytokine profile in asymptomatic and symptomatic malaria patients was evaluated. Volunteers with asymptomatic parasitaemia had a positive correlation between IL-10 and anti-SGS levels (Figure 4A. $r = 0.50$; $p = 0.0002$), but this finding was not seen in symptomatic individuals (Figure 4B. $r = 0.16$; $p = 0.17$). IFN-γ serum levels did not display significant correlation with anti-saliva antibodies in either asymptomatic (Figure 4C. $r = 0.25$; $p = 0.07$) or symptomatic (Figure 4D. $r =$

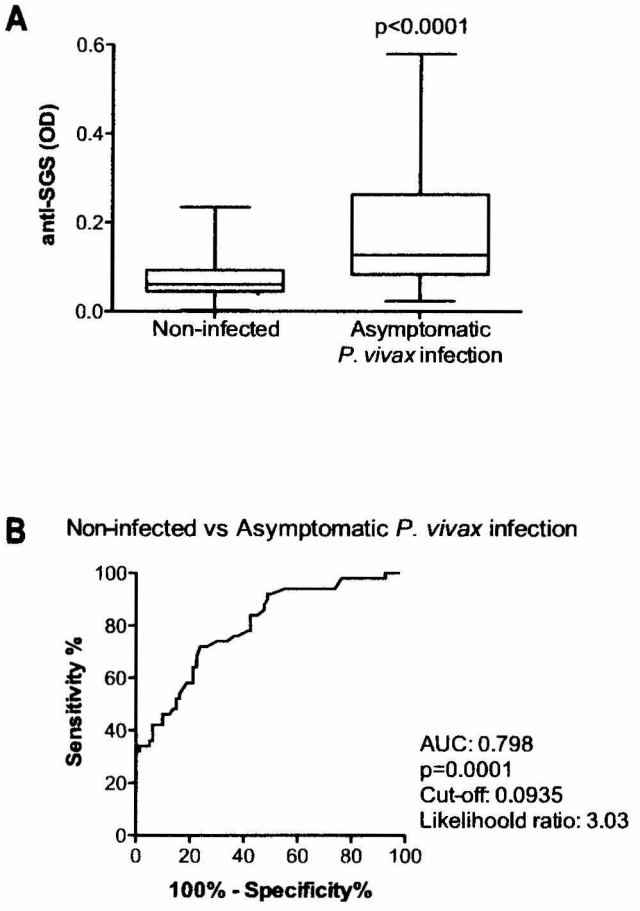

Figure 2
Anti-saliva IgG serum levels from non-infected individuals and asymptomatic malaria patients. Sera were collected from non-infected individuals (n = 80), and from patients with asymptomatic (n = 50) *P. vivax* infection. An ELISA test was performed to assess the IgG anti-*An. darlingi* SGS. (A) Box plot graphs of IgG serum levels from non-infected individuals and from patients with asymptomatic *P. vivax* infection. Lines of the boxes represent 75th percentile, median and 25th percentile of the individual average OD values; whiskers represent the maximum and minimum values. Differences between groups were tested using Mann-Whitney test. (B) ROC curve evaluating the threshold value of anti-SGS that separates non-infected individuals from asymptomatic *P. vivax* infection. Area under curve (AUC) calculated, together with the cut off value, which presents the higher likelihood ratio, and p values are plotted.

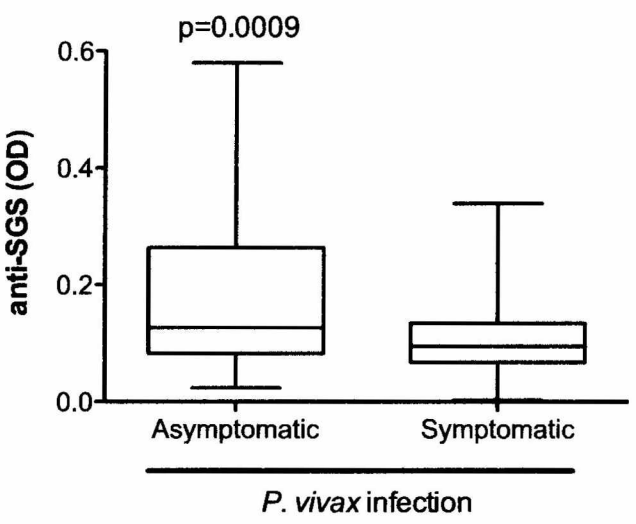

Figure 3
Serum Anti-*An. darlingi* SGS levels in patients with different clinical spectrum of *P. vivax* infection. Sera were collected from volunteers with asymptomatic *P. vivax* infection (n = 50) and from patients with symptomatic infection (n = 74). An ELISA test was performed to assess the IgG anti-*An. darlingi* SGS. Box plot graph, with lines of the boxes representing 75th percentile, median and 25th percentile of the individual average OD values; whiskers represent the maximum and minimum values. Differences between groups were tested using Mann-Whitney test; p value is plotted.

0.12; p = 0.29) patients. Moreover, a significant negative correlation between the IFN-γ/IL-10 ratio and anti-SGS levels in asymptomatic patients was noted (Figure 4E. r = -31; p = 0.03) but not in the symptomatic ones (Figure 4F. r = 0.05; p = 0.88). Thus, besides differing in anti-SGS antibody levels, asymptomatic and symptomatic *P. vivax*-infected individuals also differ in their cytokine balance. Cytokine profile may be implicated in minimizing *P. vivax* immunopathology, as individuals with asymptomatic infection presented lower IFN-γ/IL-10 ratio compared to symptomatic patients (Figure 4G; p < 0.0001). As previously described [3] and also presented in this work (Table 2), asymptomatic parasitaemia directly correlated to increased age and is more frequently observed in people residing for a long time in malaria endemic areas. In these regions, an extensive exposure to mosquito bites occurs over time. Malaria infection rates in these insects usually range from below 0.1% to 10% [23,24]. Consequently, each inhabitant is exposed to much more uninfected mosquitoes than infected ones. The recurrent exposure to mosquito bites or also to the *Plasmodium* may lead to a modification on the host immune response. It has been shown that repeated exposure to mosquito bites induces a Th1 profile in experimental models, leading to increased resistance to *Plasmodium* transmission [11]. This work shows that chronic exposure to *An. darlingi* bites relates to a reduction in the IFN-γ/IL-10 ratio not implying any causal relationship. On the other hand, as malaria clinical syndromes result from inadequate activation of pro-inflammatory cascades, oxidative stress and disturbs in immune regulation [25], this study hypothesizes that people residing in malaria endemic areas repeatedly exposed to uninfected mosquito bites over many years develop an

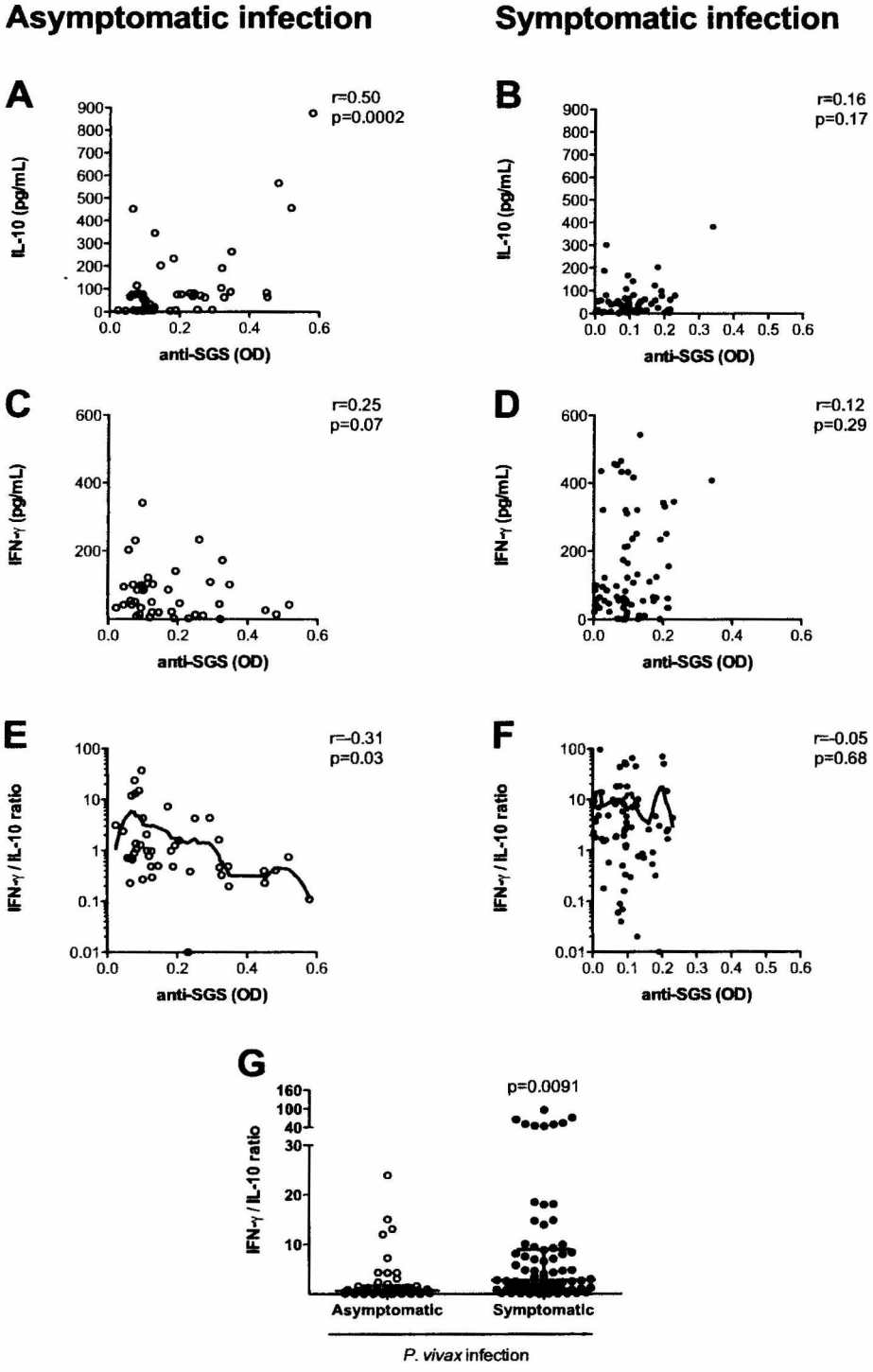

Figure 4

Correlation between cytokine plasma levels and anti-saliva IgG titers. (A) IL-10 vs. anti-SGS (OD), (B) IFN-γ, and (C) IFN-γ/IL-10 ratio vs. anti-SGS in patients with asymptomatic *P. vivax* infection. (D) IL-10 vs. anti-SGS (OD), (E) IFN-γ, and (F) IFN-γ/IL-10 ratio vs. anti-SGS in patients with symptomatic *P. vivax* infection. Fine Lowess curves are shown in (C) and (F) to evidence the trend of the data. Non-parametric Spearman test was used to verify statistical significance. (G) Comparison of IFN-γ/IL-10 ratio between volunteers with asymptomatic or symptomatic *P. vivax* infection. Mann-Whitney test was used to estimate the significance. P values, together with r values, are plotted in each graph.

efficient anti-saliva immune response, in which IL-10 may favor the production of specific antibodies. The neutralization of some vector salivary proteins may create microenvironment alterations in the site of mosquito bites that might ultimately affect the transmission of malaria. Another possibility is that mosquito bites, and also the continued exposure to *Plasmodium*, induce higher production of IL-10, which may reduce intense pro-inflammatory responses and the immunopathology of the infection. This study does not present experimental basis to indicate any direct effect of antibodies against *An. darlingi* salivary components on clinical status of *P. vivax* infected individuals. An important limitation regarding the use of anti-SGS levels as a marker for malaria infection is represented by the considerable variation of malaria transmission among different areas and also from season to season. This would make mandatory the establishment of appropriate anti-SGS cut-off level before using it as a marker for malaria. Nevertheless, once the cut-off levels are defined, the measurement of anti-SGS could serve as a very sensible indicator of this disease.

Conclusion

Through the estimation of serum anti-*An. darlingi* saliva antibody levels, it is possible to infer the probable *P. vivax* infection status as marker of disease severity of an individual from the Amazon endemic area. Moreover, this study also suggests that the clinical immunity against *P. vivax* could be associated to a specific humoral response against the salivary components. As previously described to other vector-borne diseases, such as leishmaniasis, the detection of increased levels of anti-vector saliva could be pointed as an epidemiological marker of infection and also as a suitable indicator of clinical immunity in endemic regions.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BBA designed the study, collected the serum samples, performed serology and cytokine experiments, the statistical analysis and drafted the manuscript. BCR, WPT, and LAM provided the salivary glands and helped in data analysis. ARF performed the SGS preparation and helped with the manuscript. LMAC participated in the design of the study, collected serum samples, examined the volunteers, and helped in data analysis. AB participated in the design of the study and helped in data analysis. MBN conceived of the study, participated in its design and coordination and helped in writing the manuscript. All authors have read and approved the final manuscript.

Authors' information

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5.3 MANUSCRITO III

Hepatitis B infection reduces malaria severity.

Este trabalho avalia o impacto da infecção com o vírus da hepatite B sobre a apresentação clínica da malária.

Resumo dos resultados: Indivíduos infectados pelo plasmódio e que foram expostos ao vírus da hepatite B apresentaram maior chance de serem assintomáticos (OR: 120,13; $p<0,01$) e de apresentarem parasitemia mais baixa e menores níveis de marcadores inflamatórios. Entretanto, os indivíduos co-infectados apresentaram maiores viremias. A parasitemia mostrou correlação inversa com a viremia ($r=-0,6$; $p<0.05$). Indivíduos com malária assintomática mostraram valores menores da razão IFNgama/IL10 e menor disfunção orgânica, e a hepatite B não modificou estes padrões.

Este trabalho foi submetido para revisão no periódico internacional *Clinical Infectious Diseases* (Fator de Impacto JCR 2009 = 8.195) e apresenta-se em fase de revisão por pares.

Hepatitis B Infection Reduces Malaria Severity

Running title: HBV and malaria severity

Word count abstract: 200

Word count text: 3,031

Figures/Tables: 6

Footnote Page

The authors declare that they do not have a commercial association that might pose a conflict of interest.

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Abstract

Background. Malaria endemic areas are highly endemic for hepatitis B virus (HBV) infection. Little is known about the mutual influences of such co-infection. This study aimed to verify if the HBV infection influences malaria severity.

Methods. In this study, 580 individuals from Brazilian Amazon were parasitologically screened for Plasmodium and serologically and parasitologically screened for HBV infections and followed up for 30 days for discrimination of malaria-related symptoms. Epidemiological, demographic data, and plasma cytokine profiles were studied and evaluated by multinomial logistic regression.

Results. Plasmodium-infected individuals with past or active HBV infection were more likely to be asymptomatic (OR: 120.13, $P<0.0001$), to present lower parasitemia and a decreased inflammatory cytokine profile. Conversely, co-infected individuals presented higher HBV viremia. Plasmodium parasitemia inversely correlated with plasma HBV DNA levels ($r=-0.6$; $P=0.0003$). Individuals with asymptomatic malaria showed reduced IFN- γ/IL-10 ratios, and less organ dysfunction than individuals with symptomatic malaria, with HBV infection not modifying this pattern.

Conclusions. HBV infection diminishes the intensity of malaria infection in individuals from this endemic area. This effect seems related to the cytokine balance and control of inflammatory responses. Possible immunomodulation trigged by high IL-10 in co-infected individuals may hamper the anti-HBV specific responses, favoring chronic HBV infection.

Keywords: Hepatitis B virus, Plasmodium, malaria, co-infection, cytokine.

Background

Human malaria continues to be a major health threat worldwide. Most regions highly endemic for malaria also have other important infectious diseases that may affect the malaria infection [1]. Viruses induce a robust Th1-biased immune response, which is important for *Plasmodium* clearance, but is also implicated in disease severity [2]. Most areas with malaria are highly endemic for viral hepatitis, but little is known of the affects of HBV on the clinical presentation of malaria. Experimentally intrahepatic HBV replication is inhibited by *P. yoelii* infection [3], and there is enhanced IFN-γ and IFN- α/β produced in the liver. In man positivity for the HBV surface antigen (HBSAg) did not influence mortality from *P. falciparum* [4] and HBV viraemia did not show a clear pattern in a limited number of patients during acute *P. falciparum* malaria [5]. There is no convincing evidence of the alteration of the clinical status of underlying hepatitis B-related liver disease during malaria infection. In addition, the impact of HBV infection on malaria symptoms has not been adequately addressed. Here, we report a study aimed at comparing co-infected individuals to single infections by HBV or *Plasmodium* in order to evaluate how HBV infection influences the malaria burden.

Study localities

A field study was performed between May 2006 and September 2007 in Buritis (10°12'43'' S; 63°49'44'' W), a recently urbanized municipality, and Demarcação (8°10'04.12'' S; 62°46'52.33'' W), a riverine community, both in Rondônia State, in the south-western Brazilian Amazon. Most malaria cases occur between April and September, with moderate risk of infection [6, 7]. Rondônia accounts for 19% of

malaria cases in the Brazilian Amazon (112,165 symptomatic cases in 2005), with an estimated prevalence of 8% [8]. The incidence of HBV infection was 20.4 per 100 thousands inhabitants in 2004 with a mortality rate of 7.43 per million, more than three times higher than the national mean of 2.37 [8]. Previous studies in the Brazilian Amazon have primarily tried to estimate co-infection rates[9].

Study design, sampling and data collection

Both active and passive malaria case detection strategies were performed. These included home visits in areas of high transmission, and study of individuals seeking care at the diagnostic centers of Brazilian National Foundation of Health (FUNASA). All individuals from five to seventy years, of both sexes, who resided in the endemic area for more than six months, were invited to participate. Exclusion criteria were: documented viral hepatitis other than HBV (HCV, HAV, HDV), chronic alcoholism, HIV infection, cancer or other chronic degenerative disease, and the use of drugs that induce hepatotoxicity or immunossupression. We also screened for sickle cell trait and only one individual non-infected with Plasmodium or HBV was positive. Twelve individuals withdrew consent, and were excluded from the study. All participants or those legally responsible for them gave written informed consent. Every individual was interviewed and examined by a physician, who collected 20 mL of venous blood and thick blood smears. Plasma samples and total blood were stored in cryo-tubes (Nunc, NY, USA), identified with bar-coded cryo-tags, and conserved in liquid nitrogen. Total blood samples were used for molecular diagnosis of malaria and plasma samples analyzed in our laboratory in Salvador,

Bahia, Brazil. The study was approved by the Institutional Review Board of the São Lucas University, Rondônia, Brazil.

Malaria diagnosis was performed using microscopic examination of thick smears and parasitemia (parasites/μL of blood) was calculated in positive cases. Nested PCR was performed in whole blood samples from all individuals. The laboratory personnel that performed the molecular diagnosis were blinded about the parasitological diagnosis. All positive cases were followed up for 30 days for the evaluation of malaria-related symptoms. Individuals positive for *Plasmodium* infection remaining without fever (axillary temperature >37.8°C) or chills, sweats, strong headaches, myalgia, nausea, vomiting, jaundice, asthenia, and arthralgia for 30 days were considered asymptomatic malaria cases. A case with a positive parasitological test in the presence of any symptom above was classified as symptomatic. Study individuals were classified in three groups: non-infected (n=183), and *Plasmodium*-infected (*P. vivax* and/or *P. falciparum*) either symptomless (n=202) or symptomatic (n=195). Only two cases of *P. malariae* infection were detected and these were excluded from the study.

Diagnosis of HBV infection was performed at the State Central Laboratory (LACEN) of Salvador, Bahia, using the AXSYM® automatic ELISA system (Abbott, Wiesbaden, Germany). Individuals were screened for HBSAg (and confirmatory HBSAg), total anti-HBS, total anti-HBc, anti-HBc IgM, HBeAg, and anti-HBe IgG. Hence, 326 presented no markers of HBV infection ($HBSAg^-/anti\text{-}HBS^-/anti\text{-}HBc^-$), 193 presented markers of past infection ($HBSAg^-/anti\text{-}HBS^+/anti\text{-}HBc^+$), and 61 were currently infected ($HBSAg^+/anti\text{-}HBS^-/anti\text{-}HBc^+$). All HBV infected individuals

were positive for anti-HBc. No acute HBV infection was detected, as there was no IgM to HBc antigen. Fifty-six individuals presented markers of vaccination ($HBSAg^-$ /anti-HBS^+/anti-HBc^-) and were excluded from the study. Viremia was estimated by real-time PCR (COBAS® TaqMan® HBV assay) in all individuals to confirm serological results. Although it is currently assumed that changes in plasma virus titers indicate changes in hepatic viral replication, we also evaluated HBV infected individuals for HBeAg and anti-HBe, which are more realistic indicators of viral replication.

After serology for HBV infection, 580 individuals remained in the study. Plasma measurements of aspartate amino-transferase (AST), alanine amino-transaminase (ALT), total bilirrubin, hemoglobin, fibrinogen and C reactive protein (CRP) were made at the clinical laboratory of Faculdade São Lucas and at the Pharmacy School (Federal University of Bahia, Brazil). All information regarding etiologic diagnosis, clinical symptoms and signs, demography and epidemiological issues, plasma measurements and sample storage were digitalized by a trained professional in a data bank. The physician who performed the clinical evaluations also checked the data bank. A flow chart of the study is shown in the Figure 1. The baseline characteristics of the individuals are listed in the Table 1.

Molecular malaria diagnosis

Molecular diagnosis of malaria infection was performed (in all 580 individuals studied) using nested PCR, which detailed protocol has been published [10]. To control for cross-contamination, one uninfected blood sample was included for every twelve samples processed. Fifteen percent of positive PCR samples were re-tested to confirm the amplification of plasmodial DNA. All the tests were performed and

confirmed at our main laboratory at the Centro de Pesquisas Gonçalo Moniz, Brazil.

Plasma cytokine measurement

IL-10 and IFN-γ plasma levels were measured using de Cytometric Bead Array - CBA® (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol, with all samples run in a single assay. The flow cytometric assay was performed and analyzed by a single operator, and standard curves were derived from cytokine standards. The lower limits of detection of IL-10 and IFN-γ were 2.8 and 7.1pg/mL, respectively. The experiment was repeated once to check accuracy of the measurements and data shown for each sample represent the mean values. The maximum inter-assay variation was 2.0 percent.

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. Two polytomic (multinomial) logistic regression models were carried out since the response variable (current malaria infection) was classified into three groups (non-infected, asymptomatic infection and symptomatic). In the first model, the following independent variables were included: HBV infection, malaria episodes, time residing in the area, residents per household, age and gender. The second model added plasma cytokine levels of IL-10 or IFN-γ to the previous model. The threshold values of IL-10 or IFN-γ plasma levels that discriminate asymptomatic from symptomatic malaria infection with a higher likelihood ratio were estimated using a Receiver-operator characteristic (ROC) curve analysis to categorize the individuals

according to the cytokine levels to perform the multinomial logistic regression (data not shown). The malaria parasitemia, the cytokine plasma levels, and the plasma levels of AST, ALT, total bilirrubin, fibrinogen, and CRP were compared between groups using Kruskal Wallis with Dunn's multiple comparisons. The HBV DNA plasma levels were compared between groups using the Mann Whitney test. The correlation between *Plasmodium* parasitemia and HBV viremia in co-infected individuals was checked using the Spearman test. We also plotted a non-linear curve fit to illustrate the general trend of this correlation. For each analysis, we considered as statistically significant differences whith $P<0.05$. All the statistical analysis was performed using the software STATA 9® (StataCorp, TX, USA). The graphics were plotted using GraphPad Prism 5® (GraphPad Software Inc., USA).

Results

Baseline characteristics

A total of 580 individuals, out of 681 initially approached, were included. Individuals presenting no malaria infection differed from those presenting asymptomatic or symptomatic *Plasmodium* infection with regard to all variables studied, except for gender (Table 1). A total of 254 individuals were HBV infected, with 61 being currently infected (presented HBSAg and detectable viral load) (Table 1).

Individuals not included in the study (N=101) were similar to those enrolled with regard to age, time of residence in the endemic area, number of residents per household, and number of previous malaria episodes, but were more likely to be

female (P=0.03), be negative for *Plasmodium sp.* infection on thick smear examination (P=0.001) and also negative for markers of HBV infection (P=0.02). As expected after analysis of the data in Table 1, gender did not show any association with the absence of malaria or with asymptomatic infection (Table 2).

Impact of HBV infection on malaria clinical presentation

In a multinomial logistic model, an increased number of previous malaria episodes was independently associated with no malaria infection or asymptomatic infection, while increased age was strongly related to asymptomatic infection (Table 2). On the other hand, a high number of residents per household was associated to the occurrence of symptomatic malaria infection (Table 2). Previous HBV infection was associated with no malaria infection and also asymptomatic infection (Table 2). However, current HBV infection was robustly related to asymptomatic malaria infection (Odds Ratio, 47.52; 95% CI, 11.52 to 196.08), even after adjustment for the all variables studied (Table 2).

To explore the associations described with cytokine profile, we used a multivariate model adjusting also for plasma levels of IL-10 or IFN-γ (Table 3). The association between current HBV infection with asymptomatic or no malaria infection was increased after adjustment for plasma levels of IL-10 or IFN-γ. Increased time of residence in the endemic area was no longer significantly associated with asymptomatic infection or the absence of malaria (Table 3). Nevertheless, independent associations of increased age, higher number of previous malaria, and previous or current HBV infection relations became stronger (Table 3). Furthermore, increased plasma levels of IL-10 were independently associated with asymptomatic

malaria, while higher levels of IFN-γ were related to the occurrence of symptomatic infection.

Even though the prevalence of *P. falciparum* infection in our sample was low (52 out of the 397 malaria cases), we reanalyzed all the associations considering only the *P. vivax* cases and found that the distribution of the epidemiological, demographical and immunological variables was similar to the previous analysis (data not shown). Also, the exclusion of the *P. falciparum* cases did not alter the associations between HBV infections or the cytokine plasma levels with the occurrence of asymptomatic *Plasmodium* infection (OR: 90.68, 95%CI: 18.20-451.77; OR adjusted for cytokines: 161.42, 95%CI: 21.23-1,227.31). Thus, we continue analyzing *P. vivax* and *P. falciparum* cases together.

HBV infection and laboratory assessment of malaria severity

To investigate the possible effects of active or previous HBV infection on the malaria severity, we compared malaria parasitemia between symptomatic or asymptomatic malaria cases, stratifying according to HBV status. Interestingly, in both symptomatic and asymptomatic individuals, HBV active or previous infection was linked to lower *Plasmodium* parasitemia (Figure 1A). Conversely, the number of HBV DNA copies/mL of blood in individuals co-infected with HBV and *Plasmodium* was higher than in those infected with HBV alone ($P<0.0001$; Figure 1B). In addition, these groups did not present significant differences in HBe antigen positivity ($P=0.063$; Fisher's exact test). In co-infected individuals, there was a negative correlation between *Plasmodium* parasitemia and HBV viremia (Sperman $r=-0.6$; $P=0.0003$) (Figure 1C).

Both symptomatic and asymptomatic malaria cases presented higher levels of IFN-γ (P=0.01 and P=0.023, respectively) or IL-10 (P=0.02 and P=0.001, respectively) compared to individuals not infected with the parasite (Figure 1D and Figure 1E). Individuals with asymptomatic *Plasmodium* parasitemia presented higher levels of IL-10 than those with symptomatic disease (P<0.0001; Figure 1E). Conversely, IFN-γ levels were higher in patients with symptomatic malaria compared to asymptomatic infected individuals (P<0.0001; Figure 1D). HBV was related to increased IFN-γ plasma levels in all groups analyzed (Figure 1D). On the other hand, HBV matched with increased plasma IL-10 levels only in *Plasmodium*-infected individuals (Figure 1E). Moreover, independently of HBV infection, asymptomatic *Plasmodium*-infected individuals presented higher IL-10 levels than symptomatic ones (P<0.0001; Figure 1E). IFN-γ/IL-10 ratio was higher in individuals with asymptomatic *Plasmodium* parasitemia than in symptomatic malaria patients (P<0.0001; Figure 1F). HBV was associated with higher IFN-γ/IL-10 ratio in *Plasmodium*-negative or in symptomatic malaria individuals, but not in the asymptomatic infected ones (Figure 1F).

Patients with symptomatic malaria presented higher levels of AST, ALT, total bilirubin, and CRP compared to both asymptomatic malaria and non-infected individuals (Table 4). Moreover, within symptomatic patients, previous or current HBV infection increased the levels of these parameters, besides not modifying the general prevalence of malaria-related symptoms (Table 4).

Discussion

This study is the first to provide strong evidence for the association between HBV and reduced malaria severity. *Plasmodium*-infected individuals with active or

past HBV infection were significantly more likely to be asymptomatic, to present lower parasitemia and to have a decreased inflammatory cytokine profile. Co-infected individuals presented higher HBV viremia, with *Plasmodium* parasitemia being inversely correlated to plasma HBV DNA titers. Additionally, cytokine balance seems to be linked to disease severity, as individuals with asymptomatic malaria presented a reduced IFN- γ/IL-10 ratio. Conversely, other factors besides cytokine profile must be involved in the reduced malaria severity in individuals with HBV, as the risk for asymptomatic infection was even higher when we analyzed the adjustment for plasma IL-10 and IFN-γ levels. Maybe modifications on the hepatic microenvironment during HBV infection could reduce the organ susceptibility to *Plasmodium*.

This study confirms previous observations that symptomless *Plasmodium* parasitemia correlates with increased age and longer periods of residence in malaria endemic areas [11, 12]. In addition, elevated IL-10 plasma levels correlated significantly with asymptomatic *Plasmodium* infection. Perhaps continued exposure to *Plasmodium* lead to an IL-10 mediated immunomodulatory effects that limited immunopathology. IL-10 responses have been linked to human resistance to malaria [13]. HBV infection leads to increased IFN- γ levels [14, 15]. It has been shown that IFN- γ can ultimately be important for to *Plasmodium* clearance in the liver [16], besides its early importance for malaria clinical immunity [17]. In co-infected individuals, the higher IFN- γ production could decrease parasitemia, leading to reduced malaria severity. On the other hand, *Plasmodium* infection is related to increased IL-10 plasma levels [18, 19]. Higher IL-10 production is related to reduced tissue damage in several diseases, including experimental [20] and human malaria [21, 22]. Polymorphisms associated with increased IL-10 production are related to increased severity of chronic HBV infection [23, 24]. In the present study, individuals

presenting asymptomatic malaria displayed lower IFN-γ/IL-10 ratio than their symptomatic counterparts. IL-10 may then be linked to reduced malarial liver damage as well as to increased viral load.

Other studies have addressed the association between HBV infection and *P. falciparum* but not *P. vivax* malaria. In one study, an association between HBV carriage and more severe malaria in children was observed [25]. Another paper suggested that chronic asymptomatic *P. falciparum* infection may be accompanied by sustained periods of HBV reactivation [5]. However, these data are limited in that only four patients were studied. More recently, this conclusion was reinforced by a report that HBV infection exacerbates *P. falciparum* malaria [4]. Nevertheless, that work analyzed only patients with severe *P. falciparum* infection, ignoring the effect of HBV infection on uncomplicated malaria. Our study suggests that in some individuals, HBV may worse the inflammatory cytokine parameters also altered by *Plasmodium* infection, but not influence the frequency of hospitalization the prevalence of symptoms. In other individuals, mainly those with asymptomatic malaria, the viral infection did not affect the general inflammatory profile. As the multivariate analysis revealed, other factor besides HBV infection may influence asymptomatic malaria parasitemia. Moreover, in older persons repeatedly exposed to *Plasmodium*, HBV infection does not alter malaria organ dysfunction and also reduces parasitemia. Thus, a common mechanism affecting malaria immunity is proposed, in addition to a model of exacerbation of chronic HBV infection.

We recognize a number of limitations in this study. Whilst our sample is larger than previous investigations, it still contained limited number of currently co-infected individuals (n=32). Most of our analyses were made using HBV infected individuals who had already recovered from viral infection together with those

currently infected. In addition, we do not have information regarding time since HBV cure/remission. It is unclear whether the cytokines reflect cytokine levels in the liver. Further studies are underway to address this issue. In addition, besides looking for sickle cell trait and other confounding factors, we did not screen our volunteers for some conditions, such as helminth infections or glucose-6-phosphate dehydrogenase deficiency (G6PD), important determinants of malaria infection. Helminth infection affects malaria infection [26] and reduces the associated immunopathology [27, 28]. Recently it was demonstrated that filarial infection modulates malaria-specific type-1 cytokine response in an IL-10 dependent manner [29]. Local publications indicate that the prevalence of G6PD in Rondônia is about 3.3% [30], and possibly this would not interfere with our conclusions. Finally, individuals with asymptomatic malaria parasitemia could present IL-10 or IFN-γ polymorphisms, which could in turn be considered an important bias for our interpretation. Nevertheless, the criterious analysis and detailed investigation made in this study lead us to affirm that our data are coherent and could be adequately inferred for at least this population.

In conclusion, HBV infection seems to modify the physiology of the host immune system, stimulating increased inflammatory responses, reducing *Plasmodium* parasitemia and possibly diminishing malaria burden. On the other hand, malaria infection triggers high IL-10 production that may hamper anti-HBV specific responses, favoring chronic HBV infection in co-infected individuals. These findings bring new insights on the understanding of predictive factors favouring malaria clinical immunity and also pinpoint why these two infections may commonly coexist in many regions worldwide.

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Figures (titles and legends).

Figure 1. Screening and Enrollment.

AST: aspartate amino-transferase; ALT: alanine amino-transferase; CRP: C reactive protein; PCR: polymerase chain reaction.

Figure 2. *Plasmodium* parasitemia, HBV viremia, and plasma cytokine levels.

(A) Malaria infected individuals were stratified according to the HBV status, and parasitemia levels were determined by light microscopy as described in methods. Twenty-seven asymptomatic persons, out of 202 were negative for malaria by light microscopy, but were positive for *P. vivax* by nested PCR and are not shown in the graph. (B) HBV viremia levels were estimated by real time PCR in both HBV infected (n=29) or HBV-malaria co-infected (n=32) individuals. Black filled circles indicate symptomatic patients, while clear circles represent symptomless individuals.

(C) Spearman correlation between *Plasmodium* parasitemia and HBV viremia in co-infected individuals (n=32). Non-linear curve fit was used to illustrate the general trend of the correlation. (D) IFN-γ levels, (E) IL-10 levels, and (F) IFN-γ/IL-10 ratio estimation according to HBV infection status and/or malaria clinical status. Lines represent median values. *P<0.05; **P<0.01; ***P<0.0001.

Table 1. Baseline characteristics of the subjects.

Variables	Current malaria			P value χ^2
	Non infected n= 183	Symptomatic infection n= 195	Asymptomatic infection n= 202	
	n (%)	n (%)	n (%)	
Gender				0.352
Male	88 (48.09)	107 (54.87)	99 (49.01)	
Age (years)				<0.0001
5 to 15	14 (7.65)	25 (12.82)	1 (0.50)	
16 to 30	50 (27.32)	66 (33.85)	36 (17.82)	
31 to 59	98 (53.55)	83 (42.56)	145 (71.78)	
≥ 60	21 (11.48)	21 (10.77)	20 (9.90)	
Years resident in the area				<0.0001
≤ 2	44 (24.04)	56 (28.72)	34 (16.83)	
3 to 10	20 (10.93)	41 (21.03)	19 (9.41)	
> 10	119 (65.03)	195 (50.26)	149 (73.76)	
Residents per household				<0.0001
1 to 5	141 (77.05)	113 (57.95)	147 (72.77)	
> 5	42 (22.95)	82 (42.05)	55 (27.23)	
HBV infection				<0.0001
Non-infected	89 (48.63)	140 (71.79)	97 (48.02)	
AgHBS$^-$/anti-HBS$^+$/anti-HBc$^+$	65 (35.52)	51 (26.15)	77 (38.12)	
AgHBS$^+$/anti-HBS$^-$	29 (15.85)	04 (2.05)	28 (13.86)	
Malaria diagnosis ‡				<0.0001
Negative	183 (100)	-	-	
P. vivax	-	183 (93.33)	163 (80.69)	
P. falciparum	-	08 (4.10)	32 (15.84)	
P. vivax* + *P. falciparum	-	05 (2.56)	07 (3.47)	

Malaria episodes				<0.0001
None	24 (13.11)	25 (12.82)	03 (1.49)	
1 to 4	07 (3.83)	58 (29.74)	03 (1.49)	
5 to 10	29 (15.85)	49 (25.13)	07 (3.47)	
> 10	123 (67.21)	63 (32.31)	189 (93.56)	
Plasma IL-10 (pg/mL) §				<0.0001
≤ 46	175 (95.63)	147 (75.38)	51 (25.25)	
> 46	08 (4.37)	48 (24.62)	151 (74.75)	
Plasma IFN-γ (pg/mL) §				<0.0001
≤ 198	154 (84.15)	116 (59.49)	148 (73.27)	
> 198	29 (15.85)	79 (41.51)	54 (26.73)	

Chi-square test was performed to test if the distribution of each variable varies between the groups.

Individuals presenting $AgHBS^-/anti\text{-}HBS^+/anti\text{-}HBc^+$ were considered to have previous HBV infection, while those presenting $AgHBS^+/anti\text{-}HBS^-$ were considered currently infected.

‡ Malaria diagnosis was based on light microscopy and confirmed by nested RT-PCR molecular test, as described in methods.

§ Cut-off IL-10 and IFN- γ plasma levels were determined by choosing the values, which implied the highest likelihood ratio in discriminating asymptomatic from symptomatic malaria infection using a ROC analysis.

Table 2. Adjusted multinomial logistic regression analysis of risk factors for asymptomatic malaria infection or for no malaria infection compared to symptomatic malaria.

Variables	No malaria infection	Asymptomatic malaria
	OR [95%CI]	OR [95%CI]
HBV infection		
Non-infected	1	1
AgHBS$^-$/anti-HBS$^+$/anti-HBc$^+$	2.04 [1.21; 3.42]**	2.25 [1.30; 3.92]*
AgHBS$^+$/anti-HBS$^-$	19.48 [5.45; 69.69]***	47.52 [11.52; 196.08]***
Malaria episodes		
None	1	1
1 to 4	0.37 [0.12; 1.16]	3.04 [0.44; 20.84]
5 to 10	1.81 [0.71; 4.60]	5.65 [1.05; 30.33]*
> 10	5.46 [2.27; 13.11]***	114.58 [24.55; 534.86]***
Years resident in the area		
≤ 2	1	1
3 to 10	0.55 [0.26; 1.18]	0.84 [0.35; 1.99]
> 10	1.09 [0.62; 1.90]	1.85 [0.99; 3.46]*
Residents per household		
1 to 5	1	1
> 5	0.41 [0.24; 0.68]***	0.50 [0.29; 0.86]*
Age (years)		
5 to 15	1	1
16 to 30	1.04 [0.38; 2.81]	13.03 [1.34; 126.49]*
31 to 59	1.10 [0.42; 2.90]	21.32 [2.26; 201.32]**
≥ 60	1.22 [0.39; 3.82]	14.78 [1.42; 143.69]*
Gender		
Female	1	1

Male	0.75 [0.46; 1.21]	0.78 [0.47; 1.31]

OR: Odds Ratio. CI: Confidence interval. Statistical significance was estimated through multinomial logistic regression. The symptomatic malaria infection was considered the base outcome. Adjustment was performed for all variables presented in the table. P values from statistically significant comparisons of asymptomatic malaria infection or no malaria infection to symptomatic malaria are evidenced as follows: * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

Individuals presenting $AgHBS^-/anti\text{-}HBS^+/anti\text{-}HBc^+$ were considered to have previous HBV infection, while those presenting $AgHBS^+/anti\text{-}HBS^-$ were considered currently infected.

Table 3. Multinomial logistic regression of risk factors for asymptomatic malaria infection or for no malaria infection compared to symptomatic malaria adjusted for plasma cytokine levels and other variables.

Variables	No malaria infection	Asymptomatic malaria
	OR [95%CI]	OR [95%CI]
HBV infection		
Not infected	1	1
AgHBS$^-$/anti-HBS$^+$/anti-HBc$^+$	9.95 [4.61; 21.49]***	2.43 [1.06; 5.61]*
AgHBS$^+$/anti-HBS$^-$	491.24 [92.88; 2598.16]***	120.13 [19.75; 730.64]***
Malaria episodes		
None	1	1
1 to 4	0.40 [1.09; 1.44]	4.53 [0.44; 47.96]
5 to 10	1.85 [0.65; 5.29}	4.91 [0.62; 38.78]
> 10	6.36 [2.41; 16.75}***	124.21 [18.17; 849.28]***
Years resident in the area		
≤ 2	1	1
3 to 10	0.52 [0.23; 1.21]	0.63 [0.22; 1.79]
> 10	1.03 [0.55; 1.92]	1.54 [0.72; 3.29]
Residents per household		
1 to 5	1	1
> 5	0.49 [0.27; 0.88]*	0.38 [0.19; 0.74]**
Age (years)		
5 to 15	1	1
16 to 30	1.15 [0.37; 3.62]	40.98 [1.93; 870.83]*
31 to 59	0.86 [0.28; 2.65]	118.39 [5.58; 2512.68]**
≥ 60	1.53 [0.40; 5.85]	83.26 [3.56; 1949.95]**
Gender		
Female	1	1

Male	0.89 [0.52; 1.52]	0.76 [0.41; 1.41]
Serum IL-10 (pg/mL)		
≤ 46	1	1
> 46	0.09 [0.03; 0.25]***	16.56 [7.59; 36.16]***
Serum IFN-γ (pg/mL)		
≤ 198	1	1
> 198	0.04 [0.01; 0.1]***	0.13 [0.05; 0.21]***

OR: Odds Ratio. CI: Confidence interval. Statistical significance was estimated through multinomial logistic regression. The symptomatic malaria infection was considered the base outcome. Adjustment was performed for all variables presented in the table. P values from statistically significant comparisons of asymptomatic malaria infection or no malaria infection to symptomatic malaria are evidenced as follows: * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

Individuals presenting $AgHBS^-/anti\text{-}HBS^+/anti\text{-}HBc^+$ were considered to have previous HBV infection, while those presenting $AgHBS^+/anti\text{-}HBS^-$ were considered currently infected.

Table 4. Laboratory and clinical parameters for assessment of malaria severity

Variables	Current malaria						P value
	Non-infected		Symptomatic infection		Asymptomatic infection		
	HBV -	HBV +	HBV -	HBV+	HBV -	HBV+	
	N=89	N=94	N=140	N=55	N=97	N=105	
Laboratory parameter †							
AST (U/L)							
Median	39.4	45.7	94.5	318.4	51.35	54.85	<0.0001
Range	32.7-46.0	33.6-77.2	46.8-213.6	242.6-366.1	34.43-75.2	35.5-87.7	
Median UNL (x times)	0.98	1.14	2.36	7.95	1.28	1.37	
ALT (U/L)							
Median	37.3	37.4	76.5	160.8	44.65	36.95	<0.0001
Range	29.4-45.4	30.2-52.1	38.4-134.2	114.7-216.4	32.0-65.63	27.1-56.1	
Median UNL (x times)	0.93	0.94	1.91	4.02	1.12	0.92	
Total bilirrubin (mg/dl)							
Median	0.66	0.85	1.2	2.35	0.76	0.9	<0.0001
Range	0.45-0.94	0.51-1.25	0.76-1.8	1.87-3.0	0.5-1.17	0.54-1.3	
Fibrinogen (mg/dL)							
Median	208.4	240.5	374.5	487.7	301	202.9	<0.0001
Range	191.3-293.5	201.5-343.4	234.5-485.5	386.6-502.5	205-375.2	280.3-333.7	
CRP (ng/mL)							
Median	4.7	5.65	15.5	40.85	8.15	7.35	<0.0001
Range	3.7-5.85	4.37-9.4	8.4-42.75	25.8-47.58	5.1-11.4	4.77-9.45	
Hemoglobin (g/dL)¶							
Median	13.4	13.0	9.2	8.6	11.5	11.3	0.045§
Range	9.92-14.5	9.53-14.3	6.4-11.8	5.8-12.6	9.25-13.2	8.98-14.7	
Clinical parameter (%)‡							
Fever	0	0	100	100	0	0	NS
Jaudice	0	0	22.14	25.40	0	0	NS
Splenomegaly	0	0	5.0	5.4	0	0	NS
Arthralgia	4.49	5.31	32.8	34.5	0	0	NS
Headache	2.25	7.45	96.4	87.2	0	0	0.04
Vomiting	0	0	15.7	16.4	0	0	NS

Seizure	0	0	2.14	1.82	0	0	NS
Hospitalization	0	0	7.8	7.3	0	0	NS

AST: aspartate amino-transferase; ALT: alanine amino-transferase; CRP: C reactive protein. Ranges represent interquartile interval.

UNL: Upper normal levels. Data represent the number of times a variable is higher than the standardized normal laboratory level (40U/L was used as reference value for both AST and ALT).

† Data from each laboratory parameter were compared using the Kruskal Wallis test.

§ Although the Kruskal Wallis test indicated statistical significance regarding CRP or hemoglobin levels between the groups, the Dunn's post-test did not show intra-group differences.

‡ Data from each clinical parameter was compared solely between groups in the malaria symptomatic group, using the Chi-square test.

¶ Data represent hemoglobin levels measured in 80% of individuals within each group.

Supplemental Table 1. Baseline characteristics of the subjects, considering as malaria case only *Plasmodium vivax* infection.

Variable	Current malaria			P value $\chi 2$
	Not Infected n= 183	Symptomatic infection n= 182	Asymptomatic infection n= 163	
	n (%)	n (%)	n (%)	
Sex				0.187
Male	88 (48.09)	102 (56.04)	77 (47.24)	
Age (years)				<0.0001
5 to 15	14 (7.65)	24 (13.19)	1 (0.61)	
16 to 30	50 (27.32)	63 (34.62)	25 (15.34)	
31 to 59	98 (53.55)	77 (42.31)	120 (73.62)	
≥ 60	21 (11.48)	18 (9.89)	17 (10.43)	
Time residing in the area (years)				<0.0001
≤ 2	44 (24.04)	51 (28.02)	27 (16.56)	
3 to 10	20 (10.93)	38 (20.88)	17 (10.43)	
> 10	119 (65.03)	93 (51.10)	119 (73.01)	
Residents per household				<0.0001
1 to 5	141 (77.05)	107 (58.79)	118 (72.39)	
> 5	42 (22.95)	75 (41.21)	45 (27.61)	
HBV infection				<0.0001
Not infected	89 (48.63)	132 (72.53)	74 (45.40)	
AgHBS$^-$/anti-HBS$^-$/anti-HBc$^+$	65 (35.52)	47 (25.82)	64 (39.26)	
AgHBS$^+$/anti-HBS$^-$	29 (15.85)	03 (1.65)	25 (15.34)	
Malaria episodes				<0.0001
None	24 (13.11)	25 (13.74)	03 (1.84)	
1 to 4	07 (3.83)	52 (28.57)	02 (1.23)	
5 to 10	29 (15.85)	45 (24.73)	04 (2.45)	
> 10	123 (67.21)	60 (32.97)	154 (94.48)	
Serum IL-10 (pg/mL) §				<0.0001
≤ 46	175 (95.63)	139 (76.37)	42 (25.77)	
> 46	08 (4.37)	43 (23.63)	121 (74.23)	
Serum IFN-γ (pg/mL) §				<0.0001
≤ 198	154 (84.15)	111 (60.99)	118 (72.39)	
> 198	29 (15.85)	71 (39.01)	45 (27.61)	

Chi-square test was performed to test the distribution of each variable between the groups (inter group homogeneity).

§ Cut-off IL-10 and IFN- γ plasma levels were determined by choosing the values, which implied the highest likelihood ratio in discriminating asymptomatic from symptomatic malaria infection using a ROC analysis, as described in Supplement Figure 1.

Supplemental Table 2. Multinomial logistic regression of risk factors for asymptomatic *P. vivax* infection or for no malaria infection compared to symptomatic *P. vivax* infection.

Variable	No malaria infection OR [95%CI]	Asymptomatic *P. vivax* infection OR [95%CI]
HBV infection		
Not infected	1	1
AgHBS$^-$/anti-HBS$^-$/anti-HBc$^+$	2.07 [1.22; 3.51]**	2.62 [1.45; 4.72]**
AgHBS$^+$/anti-HBS$^-$	28.96 [6.92; 121.24]***	90.68 [18.20; 451.77]***
Malaria episodes		
None	1	1
1 to 4	0.45 [0.14; 1.45]	2.92 [0.35; 24.77]
5 to 10	2.32 [0.87; 6.16]	5.11 [0.79; 32.87]
> 10	6.18 [2.49; 15.33]***	120.87 [24.29; 601.55]***
Time residing in the area (years)		
≤ 2	1	1
3 to 10	0.54 [0.25; 1.18]	1.02 [0.40; 2.60]
> 10	1.05 [0.59; 1.86]	1.91 [0.97; 3.76]
Residents per household		
1 to 5	1	1
> 5	0.41 [0.24; 0.69]**	0.52 [0.29; 0.93]*
Age (years)		
5 to 15	1	1
16 to 30	1.00 [0.37; 2.72]	8.92 [0.88; 90.10]
31 to 59	1.20 [0.45; 3.19]	19.95 [2.04; 194.97]*
≥ 60	1.40 [0.43; 4.50]	15.71 [1.44; 171.74]*
Sex		
Female	1	1
Male	0.73 [0.44; 1.18]	0.71 [0.41; 1.23]

Statistical models were estimated through multinomial logistic regression. The symptomatic *P. vivax* infection was considered the base outcome. P values from statistically significant comparisons of asymptomatic malaria infection or no malaria infection to symptomatic malaria are evidenced as follows: * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

Supplemental Table 3. Multinomial logistic regression of risk factors for asymptomatic *P. vivax* infection or for no malaria infection compared to symptomatic *P. vivax* infection, adjusted for cytokine plasma levels.

Variable	No malaria infection	Asymptomatic *P. vivax* infection
	OR [95%CI]	OR [95%CI]
HBV infection		
Not infected	1	1
AgHBS$^-$/anti-HBS$^-$/anti-HBc$^+$	10.40 [4.67; 23.18]***	2.71 [1.07; 6.90]*
AgHBS$^+$/anti-HBS$^-$	971.34 [146.99; 6418.68]***	161.42 [21.23; 1227.31]***
Malaria episodes		
None	1	1
1 to 4	0.50 [0.13; 1.87]	3.00 [0.24; 37.50]
5 to 10	2.61 [0.86; 7.90]	2.37 [0.26; 21.70]
> 10	7.67 [2.78; 21.12]***	108.74 [15.24; 775.75]***
Time residing in the area (years)		
≤ 2	1	1
3 to 10	0.49 [0.20; 1.18]	0.88 [0.29; 2.70]
> 10	1.03 [0.54; 1.96]	1.44 [0.62; 3.34]
Residents per household		
1 to 5	1	1
> 5	0.50 [0.27; 0.91]*	0.33 [0.16; 0.70]**
Age (years)		
5 to 15	1	1
16 to 30	1.09 [0.34; 3.49]	25.30 [1.16; 549.68]*
31 to 59	0.91 [0.29; 2.89]	90.68 [4.11; 1998.50]**
≥ 60	1.81 [0.46; 7.10]	82.58 [3.29; 2075.43]**
Sex		
Female	1	1

Male	0.83 [0.48; 1.45]	0.63 [0.32; 1.24]
Serum IL-10 (pg/mL)		
≤ 46	1	1
> 46	0.06 [0.02; 0.20]***	16.29 [6.78; 39.14]***
Serum IFN-γ (pg/mL)		
≤ 198	1	1
> 198	0.04 [0.02; 0.11]***	0.13 [0.05; 0.34]***

Statistical models were estimated through multinomial logistic regression. The symptomatic *P. vivax* infection was considered the base outcome. P values from statistically significant comparisons of asymptomatic malaria infection or no malaria infection to symptomatic malaria are evidenced as follows: * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

Figure 1

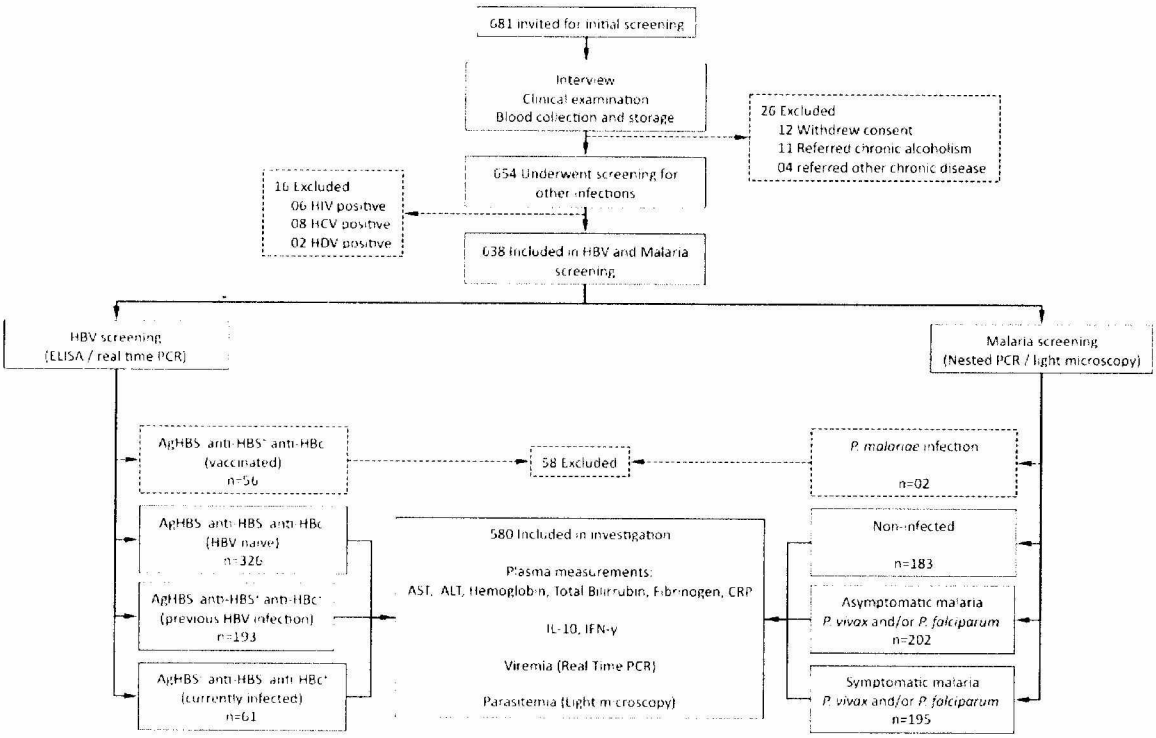
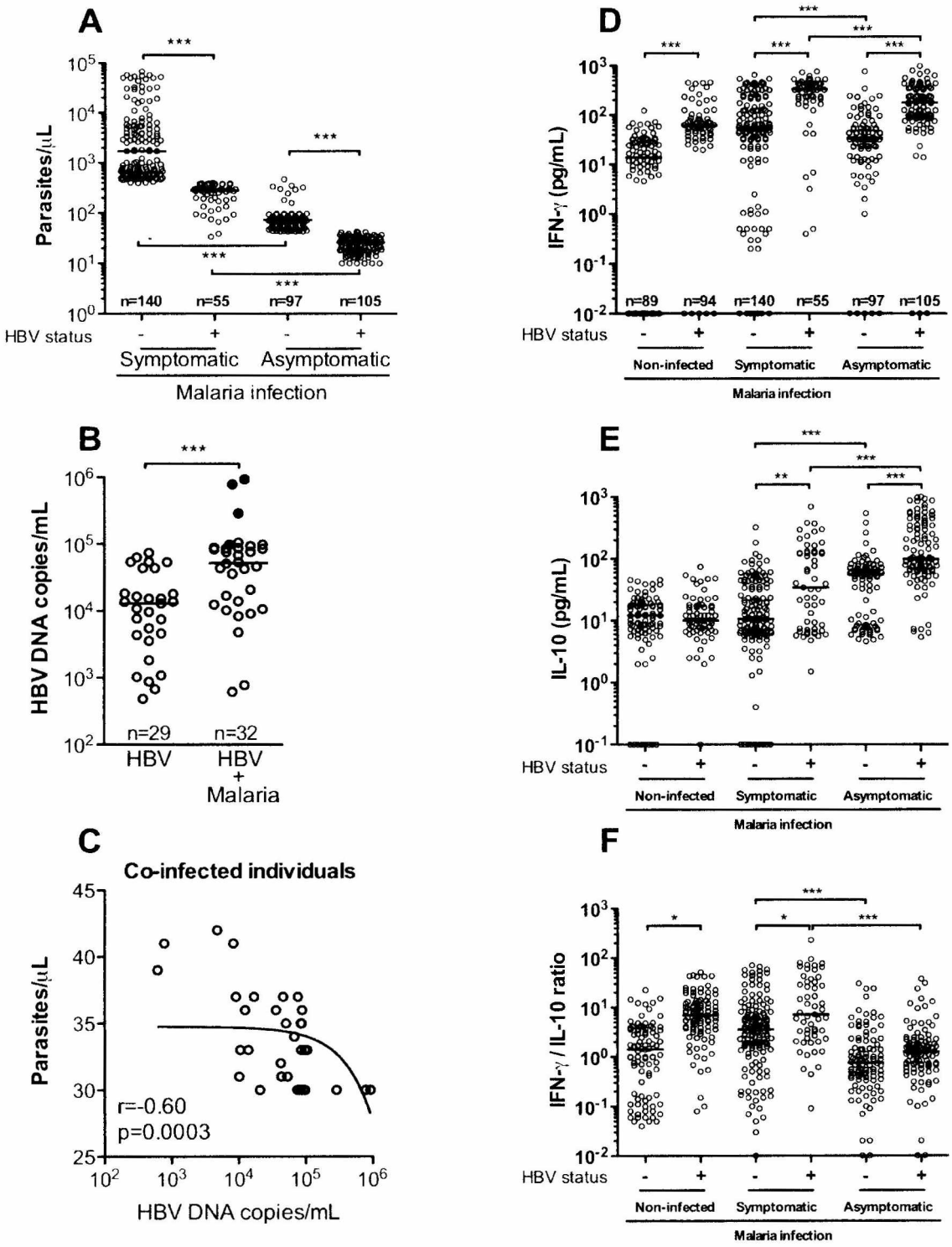

681 invited for initial screening
Interview
Clinical examination
Blood collection and storage
26 Excluded
12 Withdrew consent
11 Referred chronic alcoholism
04 referred other chronic disease
654 Underwent screening for other infections
16 Excluded
06 HIV positive
08 HCV positive
02 HDV positive
638 included in HBV and Malaria screening
HBV screening (ELISA / real time PCR)
Malaria screening (Nested PCR / light microscopy)
AgHBS⁻ anti-HBS⁺ anti-HBc⁺ (vaccinated) n=56
58 Excluded
P. malariae infection n=02
AgHBS⁻ anti-HBS⁻ anti-HBc⁻ (HBV naive) n=326
AgHBS⁺ anti-HBS⁺ anti-HBc⁺ (previous HBV infection) n=193
AgHBS⁺ anti-HBS⁻ anti-HBc⁺ (currently infected) n=61
580 Included in investigation
Plasma measurements:
AST, ALT, Hemoglobin, Total Bilirrubin, Fibrinogen, CRP
IL-10, IFN-γ
Viremia (Real Time PCR)
Parasitemia (Light microscopy)
Non-infected n=183
Asymptomatic malaria P. vivax and/or P. falciparum n=202
Symptomatic malaria P. vivax and/or P. falciparum n=195

Figure 2


A
Parasites/μL
n=140
n=55
n=97
n=105
HBV status
Symptomatic
Asymptomatic
Malaria infection
B
HBV DNA copies/mL
n=29
n=32
HBV
HBV + Malaria
C
Co-infected individuals
Parasites/μL
r=-0.60
p=0.0003
HBV DNA copies/mL
D
IFN-γ (pg/mL)
n=89
n=94
n=140
n=55
n=97
n=105
HBV status
Non-infected
Symptomatic
Asymptomatic
Malaria infection
E
IL-10 (pg/mL)
HBV status
Non-infected
Symptomatic
Asymptomatic
Malaria infection
F
IFN-γ / IL-10 ratio
HBV status
Non-infected
Symptomatic
Asymptomatic
Malaria infection

5.4 MANUSCRITO IV

Severe *Plasmodium vivax* malaria exhibits marked inflammatory imbalance.

Este trabalho caracteriza o perfil inflamatório sistêmico de pacientes com diferentes formas clínicas da infecção pelo *P. vivax*.

Resumo dos resultados: Pacientes com malária vivax grave foram mais jovem, viveram a mais tempo na are endêmica e referiram terem tido previamente mais episódios de malária em relação aos indivíduos com doença não complicada ou sintomática. Uma maior gravidade da malária foi relacionada diretamente a níveis séricos mais altos de proteína C reativa, creatinina, bilirrubinas, fator de necrose tecidual (TNF) e IFNgama, e inversamente aos níveis plasmáticos de IL-10. Tanto os parâmetros laboratoriais de disfunção orgânica quanto as citocinas pró-inflamatórias reduziram apos sete dias de tratamento nos pacientes com doença grave.

Este trabalho foi publicado no periódico internacional *Malaria Journal* (Fator de Impacto JCR 2009 = 3.00) e recebeu denominação "Highly accessed" por ter recebido 2452 acessos on-line nos primeiros três meses de publicação.

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MALARIA JOURNAL

RESEARCH Open Access

Severe *Plasmodium vivax* malaria exhibits marked inflammatory imbalance

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Abstract

Background: Despite clinical descriptions of severe vivax malaria cases having been reported, data regarding immunological and inflammatory patterns are scarce. In this report, the inflammatory and immunological status of both mild and severe vivax malaria cases are compared in order to explore immunopathological events in this disease.

Methods and Results: Active and passive malaria case detections were performed during 2007 in Buritis, Rondônia, in the Brazilian Amazon. A total of 219 participants enrolled the study. Study individuals were classified according to the presence of *Plasmodium vivax* infection within four groups: non-infected (n = 90), asymptomatic (n = 60), mild (n = 50) and severe vivax infection (n = 19). A diagnosis of malaria was made by microscopy and molecular assays. Since at present no clear criteria define severe vivax malaria, this study adapted the consensual criteria from falciparum malaria. Patients with severe *P. vivax* infection were younger, had lived for shorter time in the endemic area, and recalled having experienced less previous malaria episodes than individuals with no malaria infection and with mild or asymptomatic infection. Strong linear trends were identified regarding increasing plasma levels of C reactive protein (CRP), serum creatinine, bilirubins and the graduation of disease severity. Plasma levels of tumour necrosis factor (TNF), interferon-gamma(IFN-gamma) and also IFN-gamma/interleukin-10 ratios were increased and exhibited a linear trend with gradual augmentation of disease severity. Both laboratory parameters of organ dysfunction and inflammatory cytokines were reduced during anti-parasite therapy in those patients with severe disease.

Conclusion: Different clinical presentations of vivax malaria infection present strong association with activation of pro-inflammatory responses and cytokine imbalance. These findings are of utmost importance to improve current knowledge about physiopathological concepts of this serious widespread disease.

Background

Plasmodium vivax infection has been considered for a long time a benign and self-limited disease, mainly when compared to the burden of *Plasmodium falciparum* infection in African countries [1]. Nevertheless, *P. vivax* is responsible for up to 400 million infections each year, representing the most widespread *Plasmodium* species [2]. *Plasmodium vivax* accounts for the majority of malaria cases within the Brazilian Amazon [3], and the prevalence of asymptomatic infection is very high [4,5]. Historically, cases of complicated *P. vivax* malaria have been rare, and documented almost exclusively by case reports or small case series [6-8]. Recent evidence from larger studies performed in Melanesian populations has however reinforced the association between vivax malaria, severe complications, and death [9-11]. Severe complications associated with vivax malaria have also been reported in the Amazon region [12]. Together with rising documentation of drug resistance worldwide, the complications of *P. vivax* infection represent a global health menace which needs focused efforts to its resolution.

Major severe *P. vivax* clinical syndromes documented include important thrombocytopaenia [13,14], cerebral malaria [15,16], and acute renal [7,17], hepatic [6] and pulmonary [18,19] dysfunctions. In severe falciparum malaria syndromes, as in many other systemic

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infections, most of the pathology described seems to be a consequence of an intense inflammatory burst, favoured by a pathological activation of the immune system and cytokine release [20-22]. Despite clinical descriptions of the illness caused by *P. vivax* infection, data regarding immunological and inflammatory patterns are scarce. In the present report, inflammatory and immunological status of both mild and severe vivax malaria cases were compared in order to explore immunopathological events in this disease.

Methods

Study localities

A study investigating determinant factors for vivax malaria severity was performed during 2007 in Buritis (10°12'43" S; 63°49'44" W), a recent urbanized municipality of the Rondônia State, in the south-western part of Brazilian Amazon. Within this region, malaria transmission is unstable, with increased number of cases being detected annually between April to September, and the risk of infection is high [23], with an Annual Parasite Incidence of 77.5 per 1,000 inhabitants in 2005 [3]. The prevalence of *P. falciparum* infection in the Brazilian Amazon is 23.7% [3], and *Plasmodium malariae* case detection reaches 10% in Rondônia [24].

Participants and sampling

Active and passive malaria case detections were performed. These included home visits in areas of high disease transmission, and study of individuals who seek care at the diagnostic centers of Brazilian National Foundation of Health (FUNASA), responsible for malaria control in the country. In addition, patients admitted to the Buritis municipal Hospital (Hospital São Gabriel) presenting clinical signs of mild or complicated malaria were also included in the study. All individuals from fifteen to seventy years, of both sexes, who had been residing in the endemic area for more than six months, were invited to be included in the study. Exclusion criteria were: documented or strong clinical suspecting of viral hepatitis (HAV, HBV, HCV, HDV), chronic alcoholism, HIV infection, yellow fever, dengue, leptospirosis, tuberculosis, Hansen's disease, visceral leishmaniasis, documented or referred cancer and/or other chronic degenerative disease, sickle cell trait, and the use of hepatotoxic and immunessupressive drugs. All participants or legal responsible gave written informed consent before entering the study. This study was approved by the Ethical Committee of the São Lucas University, Rondônia, Brazil, for the human subject protocol.

Individuals were examined and interviewed and their blood samples (20 mL) were collected for serological experiments. In hospitalized participants, two venous blood collections were performed: one at the hospital admission and other seven days after malaria treatment initiation. All malaria diagnoses were performed using two methods. First, patients were screened by thick smear examination using field microscopy and the parasitaemia (parasites/uL) was quantified in positive cases. Further, nested PCR was performed in all whole blood samples to confirm the diagnosis. Two individuals presenting *P. malariae* infection and 16 people infected with *P. falciparum* (uncomplicated forms) were identified and excluded from the study. Hence, all the volunteers selected were negative for *P. falciparum* and/or *P. malariae* infection by both microscopic examination and nested PCR.

A total of 219 individuals enrolled in the study. All positive cases were followed for 30 days for the evaluation of malaria symptoms. Individuals who were positive for *P. vivax* infection and remained without fever (axillary temperature >37.8°C) and/or chills, sweats, strong headaches, myalgia, nausea, vomiting, jaundice, asthenia, and arthralgia for 30 days were considered asymptomatic *P. vivax*-infected cases. Cases showing positive parasitological tests in the presence of any symptom listed above were classified as symptomatic infections. Patients presenting any sign of acute severe organ dysfunction [25] were considered severe cases. Until today there are no clear criteria defining what a severe vivax malaria case is. Despite the absence of a consensus, this study used the previously defined criteria for severe falciparum infection [25]. Study individuals were then classified within four groups: non-infected (n = 90), asymptomatic (n = 60), mild (n = 50) and severe vivax infection (n = 19). The baseline characteristics of the volunteers are listed in the Table 1.

Nested PCR for malaria diagnosis

The molecular diagnosis of malaria infection was performed in all subjects using the nested PCR technique described previously [26,27], with minimal alterations [28]. To control for cross-contamination, one uninfected blood sample was included for every twelve samples processed. Fifteen percent of positive PCR samples were retested to confirm the amplification of plasmodial DNA. All tests were performed and confirmed at the Centro de Pesquisas Gonçalo Moniz, Salvador, Bahia, Brazil.

Plasma cytokine levels detection

Plasma levels of IL-10, IFN-gamma, and TNF were measured using the Cytometric Bead Array - CBA (BD Biosciences Pharmingen, USA) according to the manufacturer's protocol, with all samples running in a single assay. The flow cytometric assay was performed and analysed by a single operator, and standard curves were derived from cytokine standards.

Table 1 Baseline characteristics of the participants.

		Plasmodium vivax infection		
Variables	**Non-infected N = 90**	**Asymptomatic N = 60**	**Mild N = 50**	**Severe N = 19**
Male - no. (%)	39 (43.3)	30 (50.0)	22 (44.0)	10 (52.6)
Age - year *				
Median	38.0	42.0	33.0	22.0
Interquartile interval	25.0 - 51.0	32.0 - 48.2	26.7 - 48.0	16.0 - 35.0
Previous malaria episodes *				
Median	14.0	16.0	8.0	3.5
Interquartile interval	10.0 - 18.0	13.0 - 20.0	1.0 - 12	2.0 - 7.5
Years resident in the area *				
Median	11.4	12.5	7.4	3.0
Interquartile interval	3.2 - 12.8	4.2 - 14.6	0.5 - 9.2	0.5 - 5.4
Parasitaemia (parasites/uL) *				
Median	0	73 §	4,798	49,358
Interquartile interval	0	54.0 - 85.0	2,934 - 7,483	32,796 - 54,244
Haemoglobin (g/dL) *				
Median	13.2	11.5	8.9	6.4
Interquartile interval	9.2 - 14.5	9.5 - 14.2	7.3 - 12.6	5.8 - 7.4
CRP (ng/mL)*				
Median	5.65	6.6	6.5	15.3
Interquartile interval	3.7 - 9.47	4.12 - 9.35	4.9 - 8.7	11.9 - 20.65
Serum creatinine (mg/dL)*				
Median	0.85	0.9	1.1	1.7
Interquartile interval	0.7 - 1.2	0.7 - 1.2	0.7 - 1.3	1.42 - 2.45
AST (U/L)*				
Median	41.5	50.2	95.2	385.5
Interquartile interval	32.5 - 68.3	38.4 - 73.5	42.6 - 251.7	277.3 - 487.4
UNL	1.04	1.25	2.38	9.64
ALT (U/L)*				
Median	42.35	40	58.3	238.4
Interquartile interval	37.28 - 53.58	23.25 - 65.78	43.6 - 87.5	105.5 - 364.6
UNL	1.06	1	1.46	4.96
Total bilirubin (mg/dL)*				
Median	0.35	0.4	0.8	2.1
Interquartile interval	0.3 - 0.4	0.3 - 0.62	0.7 - 2.05	1.15 - 3.1
Direct bilirubin (mg/dL)				
Median	0	0.11	0.3	1.1
Interquartile interval	0 - 0	0.01 - 0.4	0 - 1.63	0 - 2.2
Indirect bilirubin (mg/dL)				
Median	0.3	0.28	0.5	1.1
Interquartile interval	0.28 - 0.37	0.2 - 0.3	0.45 - 0.72	0.6 - 1.3

CRP: C reactive protein; AST: aspartate aminotransferase; ALT: alanine amino-transferase; UNL: Upper normal levels. Data represent the number of times the median of AST or ALT is higher than the standardized normal laboratory level (40 U/L). Ordinal variables were compared using the Kruskal Wallis test with Dunn's multiple comparisons. The prevalence of male gender was compared between the groups using chi-square test. §Six out of sixty individuals with asymptomatic *P. vivax* infection were negative for malaria infection by light microscopy, but were positive for *Plasmodium vivax* infection by nested PCR. *Differences were significant between groups ($P < 0.05$).

Laboratory assessment of organ dysfunction

Plasma measurements of creatinin, aspartate amino-transferase (AST), alanine amino-transaminase (ALT), total and direct bilirubins, haemoglobin, and CRP were made at the clinical laboratory of Faculdade São Lucas, at the Pharmacy School (Federal University of Bahia, Brazil) and at the Laboratório LPC (Salvador, Bahia. Brazil).

The Hepatic-Inflammatory Parasitic score

The hepatic-inflammatory parasitic (HIP) score was created to standardize a reproducible evaluation of severity in malaria cases. This score was developed by analysing data from another study conducted in 2006 with a sample size of 580 individuals from the Buritis Municipality, Rondônia State, Brazil. This group was composed of non-infected

individuals (n = 183) and those infected with *Plasmodium* presenting malaria-related symptoms (n = 195) or asymptomatic infection (n = 202) composed this sample. In addition, this group was very similar to the one in 2007 with regard to age, gender, time of residence in endemic area and referred previous malaria episodes (data not shown). Optimal threshold plasma values of AST, ALT, total bilirubin, fibrinogen, CRP, and parasitaemia able to discriminate asymptomatic from symptomatic malaria infection were calculated using the Receiver Operator Characteristics (ROC) curves (Figure 1). For each variable measured, the cut-off values presenting the higher sensitivity and specificity, as well as the highest likelihood ratio, were established (Figure 1A-F). Further, one point was attributed to each variable that presented higher than the established cut-off value. Consequently, the minimum score was zero and maximum was five, and it reflected both parasitaemia and organ dysfunction aspects of symptomatic disease. Once the score was established, it was tested by applying to the sample constituted by the 219 participants approached in this study (Figure 1G). Additionally, the relationship between the HIP score and the IFN-gamma/IL-10 was assessed, since this ratio has been used as indicator of inflammatory activity in malaria [21,22,29].

Statistical analysis

Data were analyzed using the GraphPad Prism 5.0 (GraphPad Software Inc.). For the ordinal variables, differences between groups were calculated using the non-parametric Kruskal-Wallis test with Dunn's multiple comparisons or trend analysis. The chi-square test was used to compare differences in categorized variables. The correlations were assessed using the Spearman test. Non-linear curve fit was also plotted to illustrate the general trend of the correlations. The statistical analyses used are illustrated in each figure or table. Differences presenting $P \leq 0.05$ were considered statistically significant.

Results and Discussion

Baseline characteristics and laboratory assessment of *P. vivax* infection severity

The majority of the participants were male, with no gender differences among groups (P = 0.78). As previously described [4,28], individuals with asymptomatic *P. vivax* infection were older, had experienced more previous malaria episodes and presented lower parasitaemia than had symptomatic cases (Table 1). Patients with severe *P. vivax* infection were younger, having lived for a shorter time in the endemic area, and had experienced fewer previous malaria episodes than individuals with no malaria infection and with mild or asymptomatic infection (Table 1). Moreover, patients with severe disease

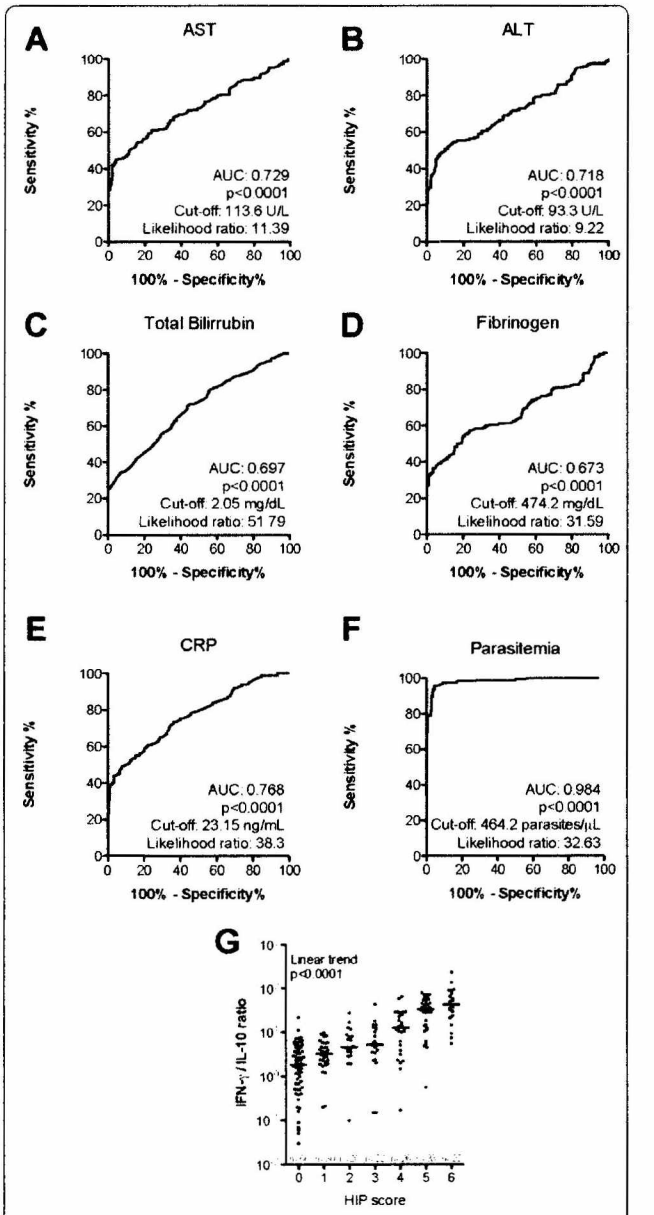

Figure 1 The Hepatic-Inflammatory Parasitic score. In a primary investigation, 580 individuals from Buritis, Rondonia, Brazil were evaluated to standardize the Hepatic-Inflammatory Parasitic (HIP) score. This sample included non-infected individuals (n = 183) and those infected with *Plasmodium* presenting malaria-related symptoms (n = 195) or asymptomatic infection (n = 202). The threshold plasma values of (A) aspartate aminotransferase (AST), (B) alanino amino-transaminase (ALT), (C) total bilirubin, (D) fibrinogen, (E) C reactive protein (CRP) and (F) parasitaemia were established in order to categorize the individuals according to the HIP score. Once the HIP score was created, it was applied in another sample from the same endemic area composed by 219 individuals: non-infected (n = 90), asymptomatic (n = 60), mild (n = 50) and severe vivax infection (n = 19). Area under the curve (AUC) was calculated, together with the cut-off value, which presents the higher likelihood ratio, and P values are plotted. The HIP score is described in Methods.

displayed higher parasitaemias than those with uncomplicated infection ($P < 0.0001$). Haemoglobin levels were also decreased in patients with severe disease ($P = 0.02$). All patients with severe disease were admitted to the municipal hospital presenting with fever, tachycardia and tachypnea. Moreover, five out of nineteen individuals with severe infection developed jaundice and six presented with splenomegaly. Six infected patients died within 72 h of hospitalization, four presenting with acute respiratory failure and two with anuric renal failure, despite the haemodynamic support and anti-parasite therapy. These severe complications have been commonly implicated as major death causes in severe vivax infections [7,18]. The other thirteen individuals with complicated disease received specific treatment with intravenous quinine and achieved total clinical recovery after 10-15 days. Clinical characteristics and outcomes of the patients with severe malaria are summarized in Table 2. All patients with mild disease recovered totally and no drug resistance was identified within individuals studied.

The further step was to assess whether the spectrum of vivax malaria clinical presentation could be associated with laboratory parameters of organ dysfunction. In a primary analysis, strong linear trends were identified regarding increasing plasma levels of CRP, serum creatinine, bilirubins and the graduation of disease severity (Table 2; $P < 0.0001$ for all trends analyzed). The individuals presenting higher HIP scores also displayed elevated IFN-gamma/IL-10 ratios (Figure 1G). These data indicate that a high grade of general inflammation-mediated systemic damage is occurring in some vivax malaria cases, explaining the severity of their clinical presentations.

Table 2 Characterization of the patients with severe vivax malaria.

			Clinical presentation at admission		*P. vivax* diagnosis		
Patient No.	**Gender**	**Age (y)**	**Major manifestation**	**Secondary manifestation**	**Nested PCR**	**Microscopy**	**Outcome**
1	M	15	Oliguria	Hypotension, splenomegaly	+	+	Recovered
2	M	17	Respiratory failure	Hypotension	+	+	Recovered
3	F	9	Respiratory failure	Hypotension	+	+	Died
4	M	21	Severe anaemia	Hypotension, splenomegaly	+	+	Recovered
5	M	22	Severe anaemia	Hypotension	+	+	Recovered
6	M	32	Anuric renal failure	Hypotension, splenomegaly	+	+	Died
7	F	41	Respiratory failure	Hypotension, Jaundice	+	+	Died
8	F	15	Severe anaemia	Splenomegaly	+	+	Recovered
9	M	15	Anuric renal failure	Hypotension, Jaundice	+	+	Died
10	M	17	Severe anaemia	Splenomegaly	+	+	Recovered
11	F	13	Jaundice	Splenomegaly	+	+	Recovered
12	F	26	Jaundice	Hypotension	+	+	Recovered
13	M	32	Respiratory failure	Hypotension	+	+	Recovered
14	F	27	Seizure	Jaundice	+	+	Recovered
15	M	42	Oliguria	Hypotension	+	+	Recovered
16	F	38	Jaundice	Hypotension	+	+	Recovered
17	F	54	Respiratory failure	Hypotension	+	+	Died
18	M	24	Severe anaemia	Hypotension	+	+	Recovered
19	M	22	Respiratory failure	Jaundice	+	+	Died

Data regarding major and secondary manifestations were obtained from medical records and/or through the clinical exam at the hospital admission. Oliguria was defined as estimated urinary output less than 400 mL/24 h and anuric renal failure as urinary output below 100 mL/24 h. Severe anaemia was defined as haemoglobin levels below 7 g/dL and jaundice by clinical exam and bilirubin levels above 2.0. Hypotension was defined as the presence of related symptoms with blood pressure below 100 x 40 mmHg. Respiratory failure was defined as tachypnea, shortness of breath, mental confusion clinical signs of hypoxaemia (central and/or peripheral cyanosis).

Inflammatory balance according to *P. vivax* infection severity

Furthermore, a possible link between the differences in clinical presentation and laboratory parameters of organ damage and specific patterns of immune responses or inflammatory mediators profile was evaluated. Plasma TNF, which is related to *P. vivax* paroxysms [30], was higher according to infection severity (Figure 2A). IFN-gamma is also implicated in both resistance to malaria [31] and disease immunopathology [32]. In the present series, IFN-gamma levels were higher in patients with increased severity (Figure 2B). Interestingly, the increasing levels of all these inflammatory markers also presented a linear trend with the gradual augmentation of infection severity ($P < 0.0001$ for each parameter). Conversely, plasma levels of IL-10, a cytokine that down-regulates inflammation, were lower with increased disease severity ($P < 0.0001$, for linear trend; Figure 2C). Thus, IFN-gamma/IL-10 ratio values were higher in patients with increased disease severity ($P < 0.0001$, for linear trend; Figure 2D).

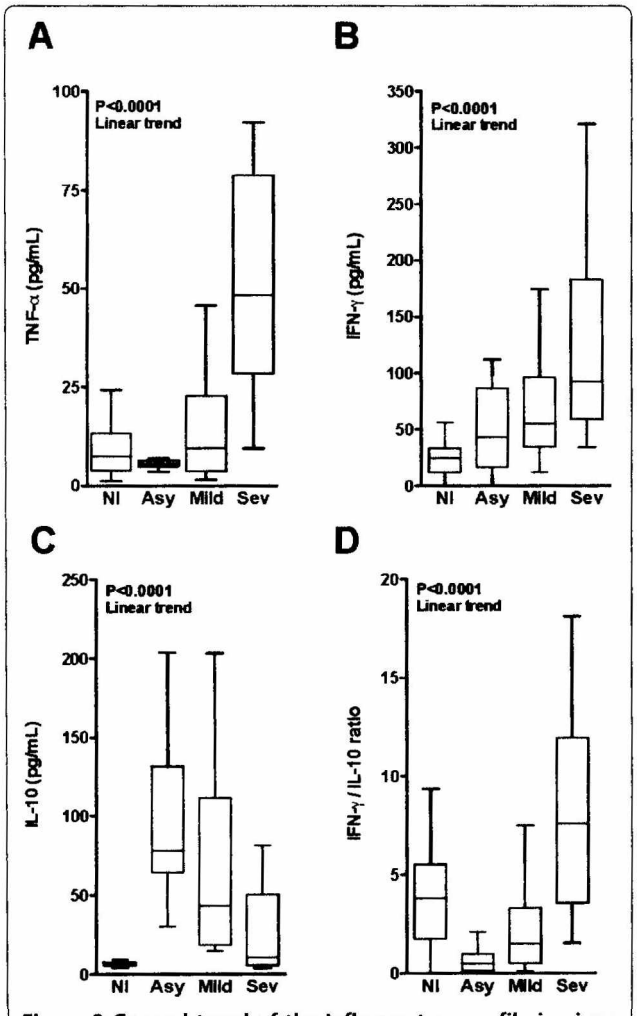

Figure 2 General trend of the inflammatory profile in vivax malaria. Plasma levels of (A) TNF, (B) IFN-gamma, (C) IL-10 and (D) IFN-gamma/IL-10 ratios were estimated in non-infected individuals and those presenting different manifestations of the vivax malaria clinical spectrum. Study participants were stratified in groups as follows: non-infected (NI; n = 90); asymptomatic infection (Asy; n = 60); mild infection (Mild; n = 50); and severe infection (Sev; n = 19). One-Way ANOVA with trend analysis was performed to check the statistical significance between the groups studied. P values are plotted in each graph.

Kinetics of inflammatory responses during the treatment of severe vivax infection

In thirteen patients, who clinically recovered out of nineteen with severe vivax infection, there was an important reduction in the levels of all laboratory parameters of organ damage screened, including plasma CRP ($P = 0.002$; Figure 3A), creatinine ($P = 0.005$; Figure 3B), ALT ($P = 0.001$; Figure 3C) and total bilirubin ($P = 0.016$;

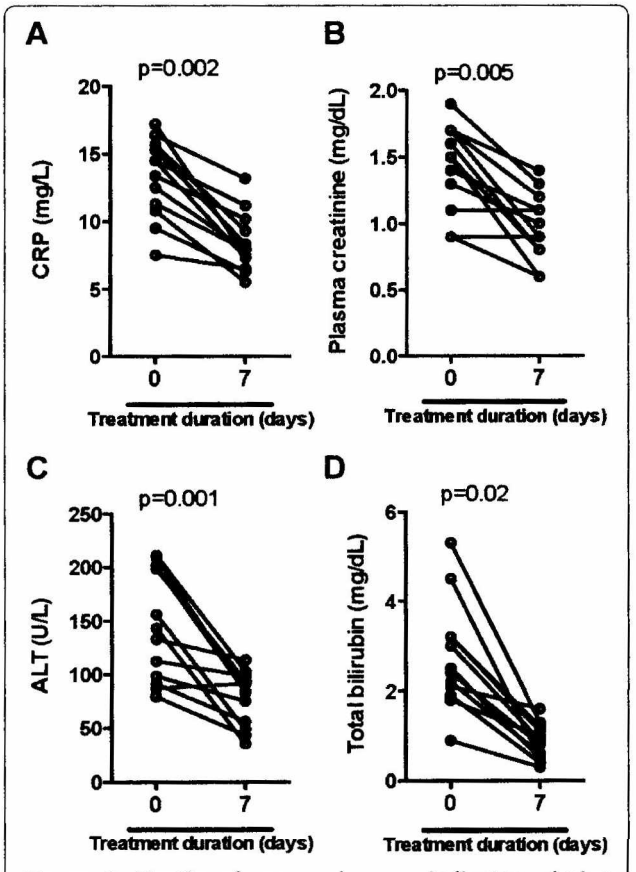

Figure 3 Kinetic of organ damage indicators during antimalarial treatment in individuals with severe vivax disease. Plasma levels of (A) CRP, (B) creatinine, (C), ALT and (D) total bilirubin were estimated before treatment (at admission to the Hospital) and after seven days of inhospital care in individuals with severe vivax infection who achieved cure (n = 13). Wilcoxon matched pairs test was performed to calculate the statistical significance. P values are plotted in each graph.

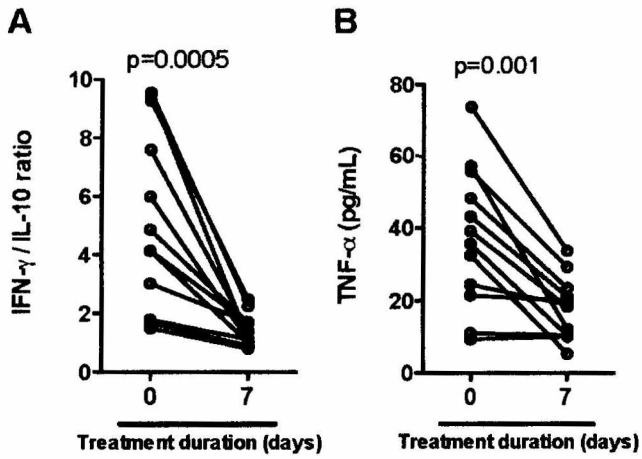

Figure 4 Kinetic of immunologic indicators during antimalarial treatment in individuals with severe vivax disease. (A) IFN-gamma/IL-10 ratios and (B) TNF plasma levels were estimated before treatment (at admission to the Hospital) and after seven days of in-hospital care in individuals with severe vivax infection who achieved cure (n = 13). Wilcoxon matched pairs test was performed to calculate the statistical significance. P values are plotted in each graph.

Figure 3D) during anti-parasite treatment. This observation suggests that clinical recovery resulted from a reduction in systemic inflammatory aggression. Regarding the immune markers of pro-inflammatory responses, an important decrease in both IFN-gamma/IL-10 ratios (P = 0.0005; Figure 4A) and TNF levels (P = 0.001; Figure 4B) was noticed during anti-malarial treatment.

Conclusions

These investigations suggest that different clinical presentations of vivax malaria infection are strongly associated with a potent activation of pro-inflammatory responses and cytokine imbalance. These results are of utmost importance to improve current knowledge about physiopathological concepts of this serious, widespread disease.

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Authors' contributions
Wrote the paper: BBA and ARF; Performed the field study and clinical examinations: BBA, SMSN and LMAC; Performed the laboratory experiments and data analysis: BBA and JC; Participated in the design of the study and helped with the manuscript: LMAC and AB; Coordinated the study helped to draft the manuscript: MBN. All authors have read and approved the final version of the manuscript.

Competing interests
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5.5 MANUSCRITO V

Plasma superoxide dismutase-1 as a surrogate marker of vivax malaria severity.

Este trabalho identifica a enzima anti-oxidante superóxido dismutase-1 como marcador potencial da malária grave causada pelo *P. vivax*.

Resumo dos resultados: O poder de predição de gravidade da enzima superóxido dismutase-1 (SOD-1) foi comparado ao poder preditor da citocina TNF-alfa, considerada um dos marcadores mais sensíveis da malária grave. A SOD-1 plasmática foi diretamente associada aos níveis de parasitemia, creatinina plasmática e alanina transaminase, enquanto que o TNF-alfa se correlacionou positivamente somente com a transaminase hepática. Os níveis plasmáticos da SOD-1 mostraram-se mais eficazes na predição das formas graves da malária do que o TNF-alfa. Tanto a SOD-1 quanto o TNF-alfa não foram eficientes em diferenciar as infecções causadas pelo *P. vivax* das causadas pelo *P. falciparum*. A SOD-1 mostrou-se então como um poderoso biomarcador de gravidade da malária.

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Plasma Superoxide Dismutase-1 as a Surrogate Marker of Vivax Malaria Severity

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Abstract

Background: Severe outcomes have been described for both *Plasmodium falciparum* and *P. vivax* infections. The identification of sensitive and reliable markers of disease severity is fundamental to improving patient care. An intense pro-inflammatory response with oxidative stress and production of reactive oxygen species is present in malaria. Inflammatory cytokines such as tumor necrosis factor-alpha (TNF-alpha) and antioxidant agents such as superoxide dismutase-1 (SOD-1) are likely candidate biomarkers for disease severity. Here we tested whether plasma levels of SOD-1 could serve as a biomarker of severe vivax malaria.

Methodology/Principal Findings: Plasma samples were obtained from residents of the Brazilian Amazon with a high risk for *P. vivax* transmission. Malaria diagnosis was made by both microscopy and nested PCR. A total of 219 individuals were enrolled: non-infected volunteers (n = 90) and individuals with vivax malaria: asymptomatic (n = 60), mild (n = 50) and severe infection (n = 19). SOD-1 was directly associated with parasitaemia, plasma creatinine and alanine amino-transaminase levels, while TNF-alpha correlated only with the later enzyme. The predictive power of SOD-1 and TNF-alpha levels was compared. SOD-1 protein levels were more effective at predicting vivax malaria severity than TNF-alpha. For discrimination of mild infection, elevated SOD-1 levels showed greater sensitivity than TNF-alpha (76% vs. 30% respectively; $p<0.0001$), with higher specificity (100% vs. 97%; $p<0.0001$). In predicting severe vivax malaria, SOD-1 levels exhibited higher sensitivity than TNF-alpha (80% vs. 56%, respectively; $p<0.0001$; likelihood ratio: 7.45 vs. 3.14; $p<0.0001$). Neither SOD-1 nor TNF-alpha could discriminate *P. vivax* infections from those caused by *P. falciparum*.

Conclusion: SOD-1 is a powerful predictor of disease severity in individuals with different clinical presentations of vivax malaria.

Citation: Andrade BB, Reis-Filho A, Souza-Neto SM, Raffaele-Netto I, Camargo LMA, et al. (2010) Plasma Superoxide Dismutase-1 as a Surrogate Marker of Vivax Malaria Severity. PLoS Negl Trop Dis 4(4): e650. doi:10.1371/journal.pntd.0000650

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Introduction

Severe malaria presents a relevant public health problem worldwide, affecting the socio-economic development of many communities. The identification of predictors of disease severity is critical to improve patient care. Most of the actual knowledge regarding the immunopathological determinants of malaria severity refers to infection caused by *Plasmodium falciparum*, but growing evidence also associates vivax malaria with severe complications [1,2]. Together with rising documentation of drug resistance worldwide, the complications of *Plasmodium vivax* infection represents a global health threat. Therefore, identifying markers of disease severity is essential to improve clinical management. Plasma TNF-alpha levels have been described as a biomarker for the estimation of disease severity for *P. falciparum* [3] and is associated with clinical severity in *P. vivax* [4] infections, but there is scarce data evaluating or validating more sensitive and reliable predictors of severe disease.

During malaria infection, reactive oxygen species (mainly superoxide anions) are produced at high levels, inducing parasite killing and tissue damage [5]. To circumvent this biological injury, the anti-oxidant enzyme Cu/Zn superoxide dismutase (SOD-1) converts these unstable free radicals into hydrogen peroxide (H_2O_2), which can be removed by the catalase and glutathione systems [6]. Studies in both mice [7] and humans [8] have correlated the SOD-1 activity with tissue damage. Therefore, investigating markers related to oxidative stress could provide useful tools to manage malaria. The present work shows that the plasma level of SOD-1 is a surrogate marker of severe vivax malaria in a population from the Brazilian Amazon, in which *P. vivax* infection is highly endemic. The performance of SOD-1 as a predictor of disease severity even surpasses that of TNF-alpha.

Author Summary

Despite being considered a relatively benign disease, *Plasmodium vivax* infection has been associated with fatal outcomes due to treatment failure or inadequate health care. The identification of sensitive and reliable markers of disease severity is important to improve the quality of patient care. Although not imperative, a good marker should have a close causative relationship with the disease pathogenesis. During acute malaria, an intense inflammatory response and a well-documented oxidative burst are noted. Among the free radicals released, superoxide anions account for the great majority. The present study aimed to evaluate the reliability of using an antioxidant enzyme, responsible for the clearance of superoxide anions, as a marker of vivax malaria severity. Thus, we investigated individuals from an Amazonian region highly endemic for vivax malaria with the goal of predicting infection severity by measuring superoxide dismutase-1 (SOD-1) plasma levels. In addition, we compared the predictive power SOD-1 to that of the tumor necrosis factor (TNF)-alpha. SOD-1 was a more powerful predictor of disease severity than TNF-alpha in individuals with different clinical presentations of vivax malaria. This finding opens up new approaches in the initial screening of severe vivax malaria cases.

Methods

Objective

The objective of this study was to test whether the plasma level of SOD-1, an antioxidant enzyme, could predict vivax malaria severity with equivalent of better efficacy compared to the currently used marker TNF-alpha.

Study design and participants

Plasma samples were obtained from individuals living in Buritis, a recently urbanized municipality in Rondônia, Brazilian Amazon, with a high risk for vivax malaria transmission [9], during June 2006 and August 2007. Active and passive malaria case detections were performed. These included home visits and study of individuals who sought care at the diagnostic center of Brazilian National Foundation of Health (FUNASA). In addition, patients admitted to the Buritis municipal Hospital with clinical signs of mild or severe malaria [10] were also asked to participate in the study. All individuals from fifteen to seventy years, of both sexes, who had been living in the endemic area for more than six months, were invited to be included in the study. The malaria diagnosis was performed using two methods (double-blinded). First, patients were screened by thick smear examination using field microscopy and the parasitaemia (parasites/uL) was quantified in positive cases. Further, nested PCR was performed in all

Table 1. Baseline characteristics of the participants.

Variables	*Plasmodium vivax* infection			
	Non-infected	Asymptomatic	Mild	Severe
	N = 90	N = 60	N = 50	N = 19
Male – no. (%)	39 (43.3)	30 (50.0)	22 (44.0)	10 (52.6)
Age – year*				
Median	38.0	42.0	33.0	22.0
Interquartile interval	25.0–51.0	32.0–48.2	26.7–48.0	16.0–35.0
Parasitaemia (parasites/uL)*				
Median	0	73	4,798	49,358
Interquartile interval	0	54.0–85.0	2,934–7,483	32,796–54,244
Haemoglobin (g/dL)*				
Median	13.2	11.5	8.9	6.4
Interquartile interval	9.2–14.5	9.5–14.2	7.3–12.6	5.8–7.4
Serum creatinine (mg/dL)*				
Median	0.85	0.9	1.1	1.7
Interquartile interval	0.7–1.2	0.7–1.2	0.7–1.3	1.42–2.45
ALT (U/L)*				
Median	42.35	40	58.3	238.4
Interquartile interval	37.28–53.58	23.25–65.78	43.6–87.5	105.5–364.6
UNL	1.06	1	1.46	4.96
Clinical presentation – no. (%)[§]*				
Splenomegaly	-	-	8 (16.0)	6 (31.6)
Hypotension	-	-	6 (12.0)	14 (73.68)
Jaundice	-	-	9 (18.0)	7 (36.8)

ALT: alanine amino-transferase. UNL: Upper normal levels. Data represent the number of times the median of ALT is higher than the standardized normal laboratory level (40U/L). Ordinal variables were compared using the Kruskal Wallis test with Dunn's multiple comparisons. The prevalence of male gender was compared between the groups using chi-square test.

*Differences were significant between groups ($P<0.05$).

§The groups were compared using chi-square test.

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whole blood samples to confirm the diagnosis. Exclusion criteria were viral hepatitis (A, B, C, and D), 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. Two individuals presenting *P. malariae* infection were identified and excluded from the study. In addition, 16 age-matched people infected with *P. falciparum* (uncomplicated forms) were invited to participate. In the last phase of the study, plasma samples from these individuals with *P. falciparum* malaria were used in order to assess if the markers compared were useful to discriminate *P. vivax* from *P. falciparum* infections.

After obtaining the parasitological diagnosis, all vivax malaria positive cases were followed for 30 days. Individuals infected with *P. falciparum* were not included in the follow up. Infected individuals who remained without any presumptive malaria symptoms were considered asymptomatic; patients presenting clinical or laboratory signs of complicated malaria [10] were considered severe cases, while those who were symptomatic without any complication were mild cases. In hospitalized participants presenting with severe disease, two plasma samples were obtained: one at the hospital admission and other seven days after malaria treatment initiation. Thus, of 415 individuals initially approached, 58 were excluded for meeting exclusion criteria, 86 withdrawn consent and 36 neglected the follow up. The sample was then composed of non-infected volunteers (n = 90) and individuals with different clinical presentations of vivax malaria: asymptomatic (n = 60), mild (n = 50) and severe infection (n = 19). The detailed clinical descriptions of the participants together with the outcomes have been already addressed by our group [11]. A summary of the baseline characteristics of the participants is illustrated in Table 1. All the malaria cases were treated by the FUNASA health care professionals according to the FUNASA standardized protocols. The flow chart of the validation study is shown in Figure S1.

Ethics statement

Written informed consent was obtained from all participants, 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.

Table 2. Primers used in Nested PCR reactions.

Primer	Oligonucleotide Sequence 5′-3′	Base Pairs
PLU5	CCTGTTGTTGCCTTAAACTTC	1,200
PLU6	TTAAAATTGTTGCAGTTAAAA	
Fal1	TTAAACTGGTTTGGGAAAACCAAATATATT	205
Fal2	ACACAATGAACTCAATCATGACTACCCGTC	
Viv1	CGCTTCTAGCTTAATCCACATAACTGATAC	120
Viv2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	
Mal1	ATAACATAGTTGTACGTTAAGAATAACCGC	144
Mal2	AAAATTCCCATGCATAAAAAATTATACAAA	

PLU: *Plasmodium sp*, Fal: *Plasmodium falciparum*, Viv: *Plasmodium vivax*, Mal: *Plasmodium malariae*.

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Nested PCR for malaria diagnosis

The molecular diagnosis of malaria was performed using nested PCR, as described previously [12]. Briefly, 300 µL of whole blood collected on EDTA was prepared for DNA extraction through the phenol-chloroform method followed by precipitation with sodium acetate and ethanol. The first PCR rDNA amplification was performed with *Plasmodium* genus-specific primers named PLU5 and PLU6. Positive samples yielded a 1,200-bp fragment, which served as template for the nested reaction. The nested PCR

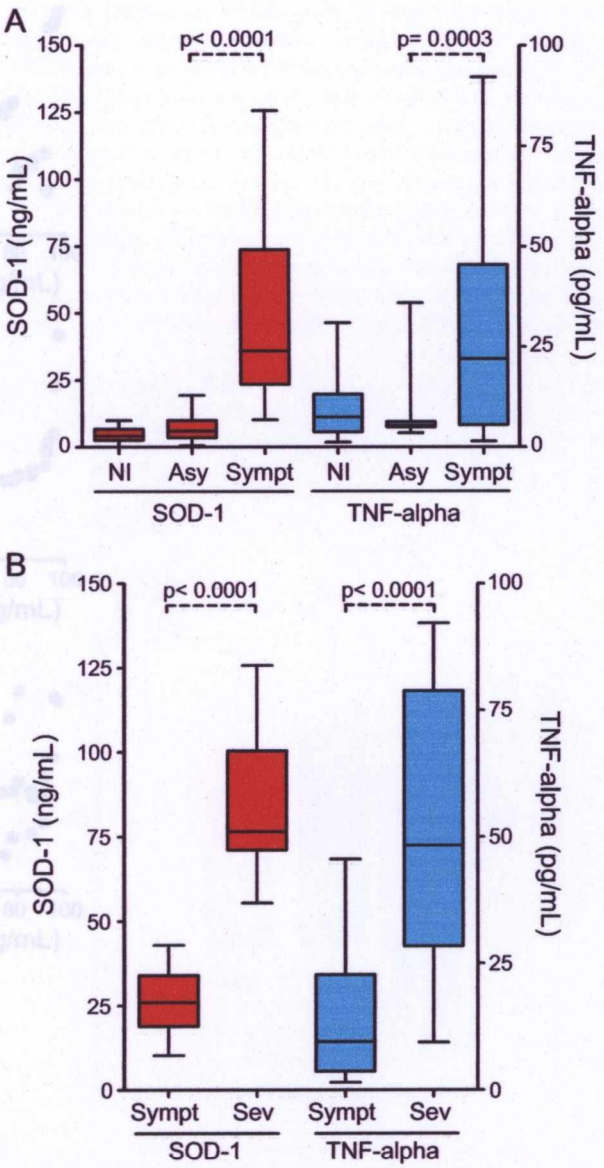

Figure 1. Plasma SOD-1 and TNF-alpha as markers of severe vivax malaria. A, SOD-1 protein and TNF-alpha plasma levels according to vivax malaria clinical severity. NI, non-infected volunteers (n = 90); Asy, asymptomatic infection (n = 60); Sympt, symptomatic infection (n = 69). Differences among the groups were calculated using the Kruskal Wallis analysis of variance with Dunn's multiple comparisons test. B, Plasma levels of SOD-1 and TNF-alpha in individuals with mild *P. vivax* infection (n = 50) compared to those with severe vivax malaria (Sev; n = 19). Boxes represent median and interquartile interval; whiskers represent maximum and minimum values. Differences were estimated using Mann-Whitney test. Lines represent median values. P values are shown in each graph.

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amplification was performed with species-specific primers for 30 cycles at annealing temperatures of 58°C for *P. falciparum* (Fal1 and Fal2 primers), and 65°C for *P. vivax* (Viv1 and Viv2 primers) or *P. malariae* (Mal1 and Mal2 primers). The fragments obtained for *P. vivax* were of 120 bp, whereas for *P. falciparum* and *P. malariae* were 205 bp and 144 bp, respectively. The oligonucleotide sequences of each primer used are listed in Table 2. The products were visualized in 2% agarose gel stained with ethidium bromide. One uninfected blood sample was included for every twelve samples processed to control for cross-contamination. Fifteen percent of positive PCR samples were re-tested to confirm the amplification of plasmodial DNA. All tests were performed and confirmed at our main laboratory at the Centro de Pesquisas Gonçalo Moniz, Brazil.

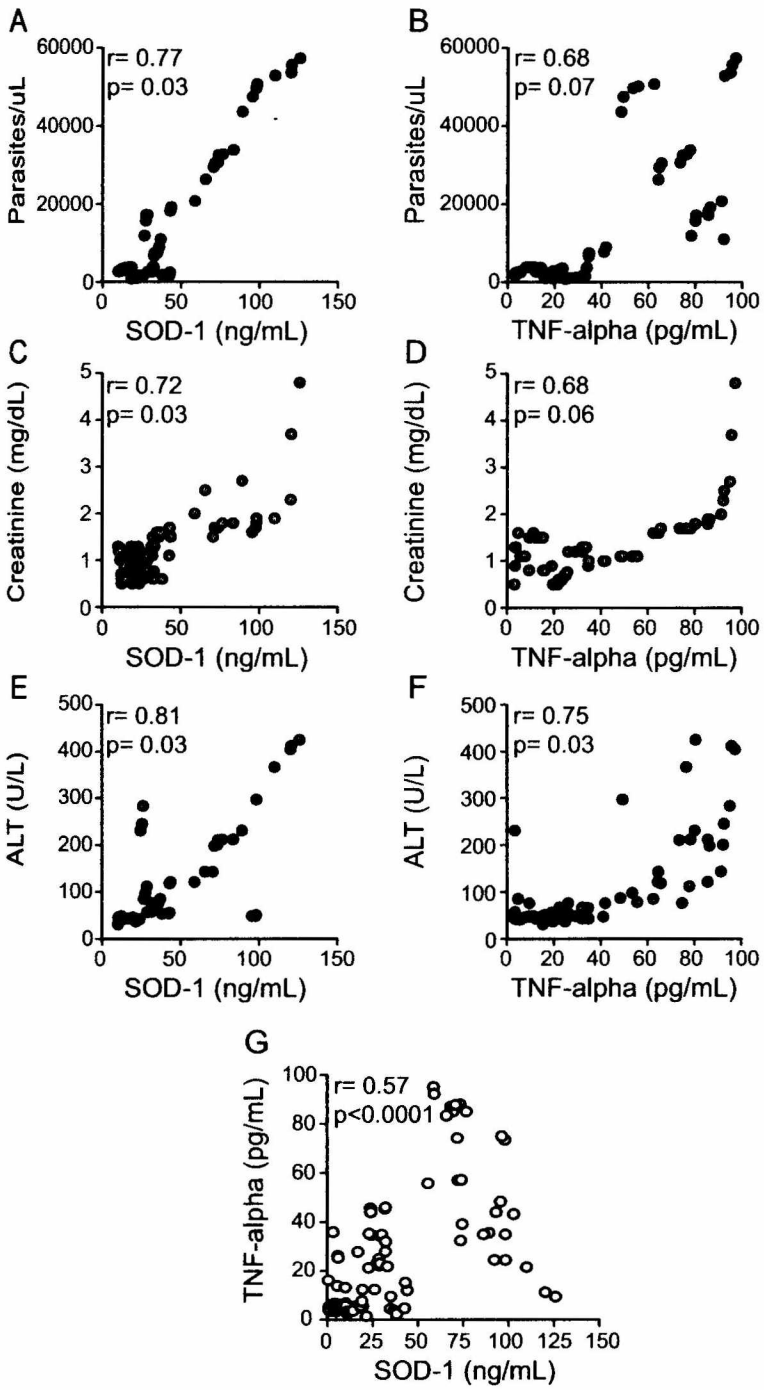

Figure 2. Correlations between plasma SOD-1 or TNF-alpha concentrations and laboratory parameters of malaria severity. Correlation of SOD-1 or TNF-alpha with several laboratory parameters in symptomatic vivax malaria patients (n = 69). Column at left (A, C and E): Correlations of SOD-1 with parasitaemia (A), plasma creatinine (C) and alanine amino-transaminase (ALT; E). Column at right: Correlations of TNF-alpha to parasitaemia (B), plasma creatinine (D) and ALT (F). Correlation between TNF-alpha and SOD-1 plasma protein levels is shown in G. The statistical significances were calculated using the Spearman test. The values of p and r are illustrated in each graph.
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Plasma cytokine measurements

Plasma levels of TNF-alpha were measured using the Cytometric Bead Array - CBA® (BD Biosciences Pharmingen, USA) according to the manufacturer's protocol, with all samples run in a single assay. The flow cytometric assay was performed and analyzed by a single operator, and standard curves were derived from cytokine standards. The minimum limit of detection was 3.7 pg/mL.

Laboratory assessment of organ dysfunction

Plasma measurements of creatinine, alanine amino-transaminase (ALT) and haemoglobin were made at the clinical laboratory of Faculdade São Lucas and at the Laboratório LPC (Salvador, Bahia. Brazil).

Plasma superoxide dismutase measurements

SOD-1 plasma concentrations were measured using the Cu/Zn Superoxide Dismutase ELISA Kit according to the manufacturer's protocol (Calbiochem, EMD chemicals, Darmstadt, Germany). Briefly, human serum was diluted 1:200 in PBS and distributed in a sensitized 96-wells plate. The samples were incubated for one hour at room temperature with HRP-conjugated anti-Cu/Zn SOD antibody. A colorimetric substrate was added for ten minutes, being the system protected from intense light. The reaction was stop and the plate read at 450nm. The SOD activity assay was performed using the Superoxide Dismutase Colorimetric Assay Kit according to the manufacturer's protocol (Cayman chemical, Ann Arbor, MI, USA). Briefly, radical detector was added to a sensitized 96-wells plate. Pre-diluted (1:50) samples were distributed in wells. The reaction was started using Xanthine Oxidase, and the plate was read after twenty minutes at 450nm. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Statistical analysis

The Kruskal-Wallis test with Dunn's multiple comparisons or linear trend analysis was used to compare SOD-1 and TNF-alpha levels according to different clinical presentations of vivax malaria infection. The Mann-Whitney test was used to verify differences between asymptomatic and symptomatic, between mild and severe vivax malaria or between *P. vivax* and *P. falciparum* infections. Correlations between SOD-1 or TNF-alpha levels and severity factors were performed using the Spearman test. Receiver-operator characteristic (ROC) curves with C-statistics were used to establish the threshold value of SOD-1 and TNF-alpha able to discriminate between mild and severe infection. A p value$<$0.05 was considered statistically significant.

Results

Increased vivax malaria severity was associated with higher plasma levels of SOD-1 ($P<0.0001$; Figure 1A and 1B), with similar trend being noted with regard to SOD activity ($P<0.01$ for linear trend; data not shown). Considering individuals with mild and severe infections together ($n=69$), increased SOD-1 protein levels were correlated with higher parasitaemia ($r=0.77$, $p=0.03$; Figure 2A), while this correlation did not reach significance for TNF-alpha ($r=0.68$, $p=0.07$; Figure 2B). In addition, splenomegaly and hypotension were more prevalent in patients with high SOD-1 and TNF-alpha levels compared to those with low levels of both factors (43.2% vs. 5.1% respectively; Fisher's test $p=0.02$). Correlation between SOD-1 protein levels and plasma creatinine measurements was $r=0.72$ ($p=0.03$; Figure 2C), while the correlation between TNF-alpha and creatinine did not achieved statistical significance in the cohort under investigation in this study ($r=0.68$, $p=0.06$; Figure 2D). Furthermore, a similar pattern was observed regarding the correlation of ALT with SOD-1 protein levels ($r=0.81$, $p=0.03$; Figure 2E) or TNF-alpha ($r=0.75$, $p=0.03$; Figure 2F). SOD-1 protein levels were also directly associated with systemic TNF-alpha ($r=0.57$, $p<0.0001$; Figure 2G). All individuals with severe disease presented with anemia at the time of hospitalization (haemoglobin mean: 6.2 ± 1.4), while in those with mild infection, only 14/50 were anemic (haemoglobin mean: 12.5 ± 2.0). In agreement with previous findings on *P. falciparum* [3] and *P. vivax* [4] infections, individuals with severe malaria displayed higher plasma levels of TNF-alpha than those with asymptomatic parasitaemia or mild disease (Figure 1B). Within the individuals presenting with severe disease who successfully recovered after in-hospital care ($n=13$), the systemic levels of both SOD-1 and TNF-alpha decreased at least two fold during the seventh day of anti-malarial treatment ($p=0.0005$ and $p=0.001$, respectively; Figure 3). We also assessed the possibility of estimating threshold levels of TNF-alpha and SOD-1 to discriminate between asymptomatic and symptomatic infection. As expected, individuals with symptomatic infection (mild or severe) presented higher levels of both TNF-alpha and

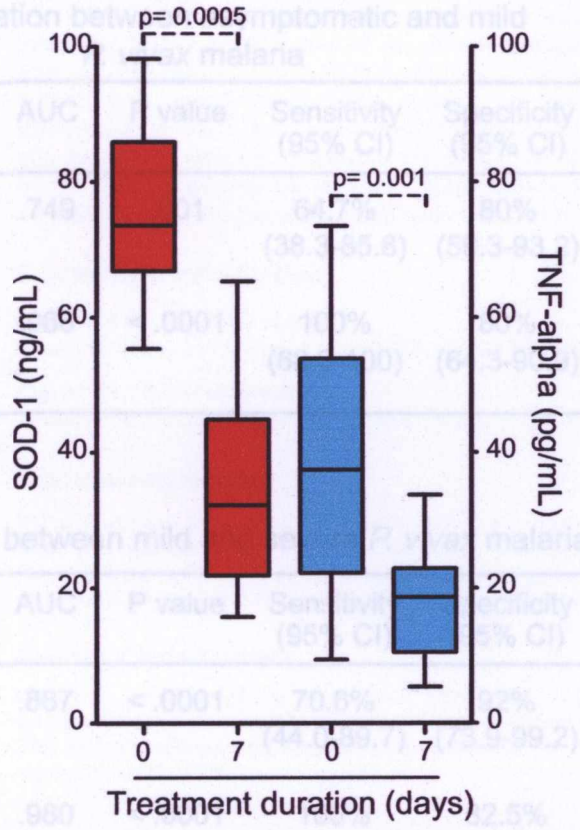

Figure 3. Effect of anti-malaria treatment on plasma concentrations of SOD-1 and TNF-alpha in individuals with severe vivax malaria. Plasma levels of SOD-1 protein (red boxes, left Y axis) and TNF-alpha (blue boxes, right Y axis) were estimated before treatment (at admission to the Hospital) and after seven days of in-hospital treatment with intravenous quinine and hemodynamic support in individuals with severe vivax infection who successful recovered ($n=13$). Boxes represent median and interquartile interval; whiskers represent maximum and minimum values. Wilcoxon matched pairs test was performed to calculate the statistical significance. P values are plotted in each graph.

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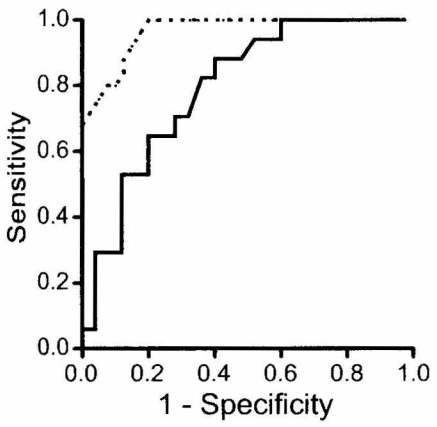
SOD-1 than those who were symptomless (Figure 1A). SOD-1, however, was a better marker than TNF-alpha (Figure 4A). Moreover, TNF-alpha and SOD-1 levels were elevated in individuals with severe disease compared to mild disease (Figure 1B). SOD-1 was also more powerful than TNF-alpha in predicting severe disease (Figure 4B). In an attempt to address if the plasma levels of both SOD-1 and TNF-alpha were useful to discriminate *P. vivax* from *P. falciparum* malaria, we compared plasma samples from individuals presenting with mild symptomatic vivax malaria and age matched individuals with symptomatic *P. falciparum* infection. Neither SOD-1 nor TNF-alpha could differentiate between the infections (Figure 5).

Discussion

This study is the first to examine the use of plasma SOD-1 levels as a surrogate marker of *P. vivax* malaria severity. SOD-1 is an important participant in the oxidative stress responses [6]. It has been implicated in several other diseases and infections [13–17], and its plasma levels could be a sensitive indicator of inflammatory processes. More recently, SOD-1 has been found to play a deleterious role in cutaneous leishmaniasis, as the interferon-beta inhibition of leishmanicidal activity was mimicked by SOD-1 and antagonized by either pharmacological or small interfering RNA-mediated inhibition of SOD-1 [18]. SOD-1 levels were much more effective in predicting vivax malaria severity than TNF-alpha, a major cytokine related to malaria clinical severity in *P. vivax* infections [19]. For discrimination of mild infection, the use of SOD-1 improves the correct case detection by more than 45% compared with the use of TNF-alpha, in addition to being a better identifier of negative cases. Furthermore, SOD-1 has a higher sensitivity than TNF-alpha in predicting severe vivax malaria, indicating also a higher likelihood ratio to discriminate this clinical condition. This suggests that SOD-1 can serve as an additional and innovative tool in the clinical approach to *P. vivax* malaria cases. The measurements of both SOD-1 and TNF-alpha in the plasma samples are performed using simple ELISA-based kits. It is possible then that costs may be similar depending on the demand. Measuring SOD-1 levels could be used in two situations: (i) identification of patients with severe disease before the development of fatal outcomes and (ii) monitoring the success of therapy and clinical recovery. The viability of applying this methodology

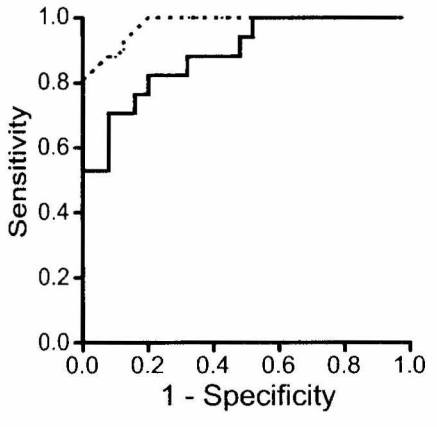
A



Discrimination between asymptomatic and mild *P. vivax* malaria

	AUC	P value	Sensitivity (95% CI)	Specificity (95% CI)
—TNF-alpha	.749	.001	64.7% (38.3-85.8)	80% (59.3-93.2)
----SOD-1	.966	< .0001	100% (68.3-100)	80% (64.3-90.9)

B



Discrimination between mild and severe *P. vivax* malaria

	AUC	P value	Sensitivity (95% CI)	Specificity (95% CI)
—TNF-alpha	.887	< .0001	70.6% (44.0-89.7)	92% (73.9-99.2)
----SOD-1	.980	< .0001	100% (87.4-100)	82.5% (67.2-92.7)

Figure 4. Effectiveness of plasma concentrations of SOD-1 and TNF-alpha measurements as markers of vivax malaria severity. A, ROC curves of SOD-1 (dashed line) and TNF-alpha (solid line) plasma levels for discriminating asymptomatic infection from mild *P. vivax* malaria cases. B, ROC curves of SOD-1 (dashed line) and TNF-alpha levels (solid line) for discriminating severe from mild *P. vivax* malaria cases. C-statistics are illustrated in the tables and were used to verify the validation of the ROC curves and the predictive power of each biomarker. AUC, area under the curves; CI, confidence interval.

doi:10.1371/journal.pntd.0000650.g004

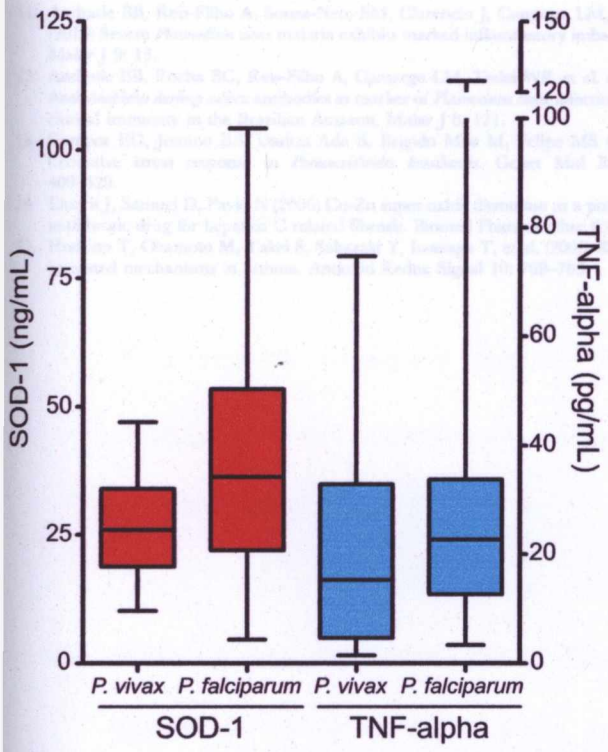


Figure 5. Plasma concentrations of SOD-1 and TNF-alpha during P. vivax and P. falciparum infections. Plasma levels of SOD-1 (red boxes) and TNF-alpha (blue boxes) were measured in patients with mild *P. vivax* (n = 50) or mild *P. falciparum* (n = 16) malaria. Boxes represent median and interquartile interval; whiskers represent maximum and minimum values. The differences between *P. vivax* and *P. falciparum* infections were not significant when compared by the Mann-Whitney test ($p>0.05$).
doi:10.1371/journal.pntd.0000650.g005

in the clinical practice will depend on its priority status in a diagnostic algorithm.

Whether this anti-oxidant enzyme could be used as a marker of disease severity in *P. falciparum* infections was not evaluated here, and should be tested in future investigations. However, in individuals presenting with mild disease, plasma SOD-1 levels could not differentiate between *P. vivax* and *P. falciparum* infections. This suggests that these two parasites may share more similar pathogenetic mechanisms than previously realized.

SOD-1 represents an important defense against oxidative stress within a cell [16,17]. Furthermore, superoxide radicals are the main ROS produced during acute malaria [5]. The role of SOD-1 in vivax malaria could be either protective or deleterious with regard to the infection outcome. SOD-1 levels may be a reflection of an active injury mechanism or, alternatively, may indicate a counter-regulatory response to the generation of superoxide radicals. Supplementation of SOD-1 protects endothelial cells against the *P. falciparum*-induced oxidative response and apoptosis *in vitro* [19]. Nevertheless, during experimental malaria, mice overexpressing SOD-1 develop oxidative injury associated with increased vulnerability to *P. berghei* [7]. Patients with acute non-complicated *P. falciparum* or *P. vivax* malaria have less catalase activity then non-infected individuals but higher SOD activity [8]. Reduced catalase activity together with increased SOD activity may result in the accumulation of H_2O_2, the release of hydroxyl radicals and increased tissue damage during severe malaria.

Although investigations analyzing more patients with broader clinical outcomes are necessary, SOD-1 plasma protein levels seems to represent a useful marker in predicting vivax malaria severity based on the oxidative response status.

Limitations

This study illustrates the possibility of using SOD-1 levels as a severity biomarker in human *P. vivax* malaria and highlights the likelihood of exploring the future use of the plasma SOD-1 levels as an effective marker of malaria severity. To validate our results, studies investigating samples from different endemic areas are crucial, as local health conditions such as co-infections may limit the effective use of a biomarker. A possible advantage of measuring SOD-1 levels as part of the clinical management in endemic areas cannot be assumed from our results. The use of the SOD-1 as a reliable marker also depends on future field interventions in which the pre-test prediction and cost-effectiveness should be considered. In addition, the specific role of SOD-1 in the immunopathogenesis of severe vivax malaria was not explored in this study and is still being addressed by our group.

Supporting Information

Checklist S1 STARD checklist.
Found at: doi:10.1371/journal.pntd.0000650.s001 (0.08 MB PDF)

Figure S1 STARD flowchart.
Found at: doi:10.1371/journal.pntd.0000650.s002 (0.02 MB PDF)

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Author Contributions

Conceived and designed the experiments: BBA LMAC AB MBN. Performed the experiments: BBA ARF SMSN IRN. Analyzed the data: BBA ARF. Contributed reagents/materials/analysis tools: AB. Wrote the paper: BBA ARF MBN.

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5.6 MANUSCRITO VI

Heme impairs PGE_2 and TGF-β1 production by human mononuclear cells via Cu/Zn superoxide dismutase: insight into the pathogenesis of severe malaria

Este trabalho explora a possível função da enzima superóxido dismutase-1 na desregulação das respostas anti-inflamatórias que resulta na forma grave da malária causada pelo *P. vivax*.

Resumo dos resultados: Pacientes com malária vivax grave apresentaram maiores níveis de heme e indícios de mais hemólise, além de menores níveis de prostraglandina E2 (PGE2) e TGF-beta do que os com doença não complicada. Houve uma correlação inversa entre os níveis de SOD-1 e PGE2 ou TGF-beta nos pacientes graves. O heme livre regulou a liberação de PGE2 e TGF-beta por monócitos humanos. Experimentos com RNA de interferência revelaram que o efeito do heme depende da sua ligação à molécula CD14 e da ativação da SOD-1.

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ihed June 18, 2010, doi:10.4049/jimmunol.0904179

Heme Impairs Prostaglandin E_2 and TGF-β Production by Human Mononuclear Cells via Cu/Zn Superoxide Dismutase: Insight into the Pathogenesis of Severe Malaria

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In many hemolytic disorders, such as malaria, the release of free heme has been involved in the triggering of oxidative stress and tissue damage. Patients presenting with severe forms of malaria commonly have impaired regulatory responses. Although intriguing, there is scarce data about the involvement of heme on the regulation of immune responses. In this study, we investigated the relation of free heme and the suppression of anti-inflammatory mediators such as PGE_2 and TGF-β in human vivax malaria. Patients with severe disease presented higher hemolysis and higher plasma concentrations of Cu/Zn superoxide dismutase (SOD-1) and lower concentrations of PGE_2 and TGF-β than those with mild disease. In addition, there was a positive correlation between SOD-1 concentrations and plasma levels of TNF-α. During antimalaria treatment, the concentrations of plasma SOD-1 reduced whereas PGE_2 and TGF-β increased in the individuals severely ill. Using an in vitro model with human mononuclear cells, we demonstrated that the heme effect on the impairment of the production of PGE_2 and TGF-β partially involves heme binding to CD14 and depends on the production of SOD-1. Aside from furthering the current knowledge about the pathogenesis of vivax malaria, the present results may represent a general mechanism for hemolytic diseases and could be useful for future studies of therapeutic approaches. ***The Journal of Immunology*****, 2010, 185: 000–000.**

Severe malaria is a highly lethal condition and a major health threat in many tropical countries. Multiple factors have been implicated in the pathogenesis of the severe complications of this condition, such as uncontrolled cytokine production (1, 2), hemolysis (3), and erythropoiesis suppression (4). Severe malaria was firstly described as originating from *Plasmodium falciparum* infection (5), but severe cases, including those with lethal outcomes, have also been observed from *Plasmodium vivax* infections (6–8). One of the major factors thought to be involved in sustaining systemic inflammation is the release of free heme, as a consequence of hemolysis inherent to the life cycle of *Plasmodium* within RBCs (9). Recently, heme has been implicated in the pathogenesis of severe forms of malaria in mice (10, 11). Under homeostasis, the heme released from hemoproteins such as cell-free hemoglobin (Hb) is scavenged by plasma proteins such as hemopexin or albumin as well as by lipoproteins (12). However, these proteins can be depleted during severe hemolytic conditions, such as associated with *Plasmodium* infection (13). This leads to the accumulation of free Hb tetramers in the plasma (14), which dissociate spontaneously into dimers. In the presence of reactive oxygen species (ROS) or other free radicals, cell-free Hb dimers are readily oxidized into methemoglobin, releasing their heme prosthetic groups (12). As a consequence, in malaria and other hemolytic disorders, the concentrations of heme can reach levels of up to 50 μM in the bloodstream (15), which can trigger an intense oxidative burst and unspecific tissue damage (11). Moreover, a crystal form of heme molecules produced by *Plasmodium sp.*, and referred to as hemozoin, also acts as a proinflammatory agonist and thus could be associated with the development of severe forms of malaria (16–18). Hemozoin inhibits PGE_2 production in both mice (19) and humans (20, 21), and there is an inverse relationship between PGE_2 and blood mononuclear cell cyclooxygenase-2 with disease severity in children with *P. falciparum* malaria (22). Until now there is no clear description of the effect of free heme on the PGE_2 production.

During malaria infection, superoxide anions are thought to be the main form of ROS produced (23). In this context, the antioxidant enzyme Cu/Zn superoxide dismutase (SOD-1) is activated and may display an important role in the pathological oxidative injury. Notwithstanding, SOD-1 has been linked to an increased inflammatory activity by amplifying TNF-α production on macrophages (24). In addition, overexpression of SOD-1 increases NF-κB–related rapid responses, such as immune response and antiapoptosis factors (25). Therefore, studies have correlated SOD-1 activity with

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Abbreviations used in this paper: 7-AAD, 7-aminoactinomycin D; A, asymptomatic; ALT, alanine aminotransferase; CoPPIX, cobalt protoporphyrin IX; CRP, C-reactive protein; DETC, diethyldithiocarbamate; Hb, hemoglobin; HO-1, heme oxygenase-1; M, mild; NAC, *N*-acetyl-L-cysteine; NI, noninfected individual; PPIX, protoporphyrin IX; ROS, reactive oxygen species; S, severe; siRNA, small interfering RNA; SnPPIX, Tin protoporphyrin IX; SOD-1, Cu/Zn superoxide dismutase.

tissue damage (26, 27). More recently, SOD-1 has been found to play a deleterious role in protozoan infectious diseases such as leishmaniasis (28). Mice overexpressing SOD-1 develop oxidative injury associated with an increased vulnerability to *Plasmodium berghei* (26). Recent investigations from our group also indicate that SOD-1 is a surrogate marker of severe vivax malaria with a better predictive power than TNF-α (29). Although intriguing, the specific link between the free heme release from Hb, expression of SOD-1, and the severity of malaria has never been addressed. In this study, we investigate the relation of free heme and the suppression of anti-inflammatory mediators such as PGE_2 and TGF-β in human vivax malaria. We demonstrate that the heme effect on the impairment of regulatory responses partially involves heme binding to CD14 and depends on the production of SOD-1. Aside from furthering the current knowledge about the pathogenesis of vivax malaria, the present results may represent a general mechanism for hemolytic diseases and could be useful for future studies of therapeutic approaches.

Materials and Methods

Reagents

The RPMI 1640 medium and the L-glutamine, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Nutridoma-SP was obtained from Roche (Indianapolis, IN). The SOD-1 Protein ELISA kit was purchased from Calbiochem (San Diego, CA). TGF-β1 (DuoSet kit) was from R&D Systems (Minneapolis, MN). The SOD-1 activity assay and PGE_2 enzyme-linked immunoassay Kits were obtained from Cayman Chemical (Ann Arbor, MI). The superoxide dismutase inhibitor (diethyldithiocarbamate [DETC]), *N*-acetyl-L-cysteine (NAC), $FeSO_4$, endotoxin-free delipidated BSA, LPS, and apotransferrin were purchased from Sigma-Aldrich (St. Louis, MO). Heme and protoporphyrin IX (PPIX) were obtained from Frontier Scientific (Logan, UT). Porphyrins were dissolved in 0.1 N NaOH, diluted in RPMI 1640, and filtered. Stock solutions from porphyrins and heme were prepared in the dark immediately before being used to avoid free radical generation. Heme used contained <0.01 endotoxin units (<1 pg) in 200 μM heme. Purified bovine liver catalase (35 U/mg) was from Boehringer (Mannheim, Germany). 7-Aminoactinomycin D (7-AAD) and Annexin V-labeled Ab were purchased by BD Biosciences (San Jose, CA). The anti-CD14 Ab (3C10) was provided by Dr. D. Golenbock (University of Massachusetts, Amherst, MA) and Dr. R. Gazzinelli (Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Brazil).

Field study design and sampling

Plasma samples were obtained from a previous survey (8), which was part of an effort from our group to study predictive factors of human *P. vivax* malaria severity. The survey studied individuals living in Buritis, Rondônia, in the Brazilian Amazon, during 2006 and 2007. Active and passive malaria case detections were performed. Moreover, patients admitted to the Buritis Municipal Hospital with clinical signs of mild or severe malaria (5) were also asked to participate in the study. All individuals from 15 to 70 y, of both sexes, who had been living in the endemic area for >6 mo, were invited to be included in the study. The malaria diagnosis was performed using microscopy and confirmed by nested PCR. Exclusion criteria were as follows: documented or strong clinical suspicion of viral hepatitis (A, B, C, and D), chronic alcoholism, HIV type 1 infection, yellow fever, leptospirosis, cancer and chronic degenerative diseases, sickle cell trait, and the use of hepatotoxic or immunosuppressant drugs. Two individuals presenting *Plasmodium malariae* infection and 16 with *P. falciparum* infection were identified and excluded from the study. The separation of plasma and RBCs was performed within 5 min after phlebotomy in specialized laboratory facilities built inside the diagnostic centers and the municipal hospital exclusively for this study.

After obtaining the parasitological diagnosis, all vivax malaria positive cases were followed for 30 d. Infected individuals who remained without any presumptive malaria symptoms were considered asymptomatic; patients presenting clinical or laboratory signs of complicated malaria (5) were considered severe cases, whereas those who were symptomatic without any complication were mild cases. In hospitalized participants presenting with severe disease, two plasma samples were obtained: one at the hospital admission and the other 7 d after malaria treatment initiation. The sample was then composed of noninfected volunteers (n = 90) and individuals with different clinical presentations of vivax malaria: asymptomatic (n = 60), mild (n = 50), and severe infection (n = 19). The detailed clinical description of the participants together with the outcomes has been already addressed by our group (8). A summary of the baseline characteristics of the participants is illustrated in Table I. All the malaria cases were treated by the health-care professionals, according to the standardized protocols from Fundação Nacional da Saúde (Ministério da Saúde, Brazil). Written informed consent was obtained from all participants prior to enrolling in the study. The project was approved by the Institutional Review Board from the Faculdade de Medicina, Faculdade São Lucas (Porto Velho, Brazil), where the field study was performed.

Cell culture

RBC-free PMBCs were isolated from healthy donors through Ficoll gradient centrifugation, treatment with Ammonium-Chloride-Potassium lysis buffer, and cultivated (10^6 cells/well) in serum-free RPMI 1640 supplemented with 1% Nutridoma-SP, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were treated with heme (3, 10, or 30 μM) for 6, 24, and 48 h and/or with $FeSO_4$ (30 μM), PPIX (50 μM), Sn PPIX (SnPPIX; 50 μM), Co PPIX (CoPPIX; 50 μM), albumin (100 μg/ml), apotransferrin (100 μg/ml), DETC (2 mM), NAC (20 mM), or catalase (1 KU/ml) for 48 h. In some assays, cells were preincubated for 1 h with anti-CD14 Ab or IgG isotype controls (10 μg/ml). All of the conditions were also run in the presence or absence of polymixin B (New Bedford Laboratories, Bedford, OH), with no significant differences between the values obtained, except for those stimulated with LPS (data not shown). In some experiments, a possible toxicity induced by heme was assessed by flow cytometry analysis of cells stained with 7-AAD and Annexin V (Pacific orange)-labeled Ab after 6, 24,

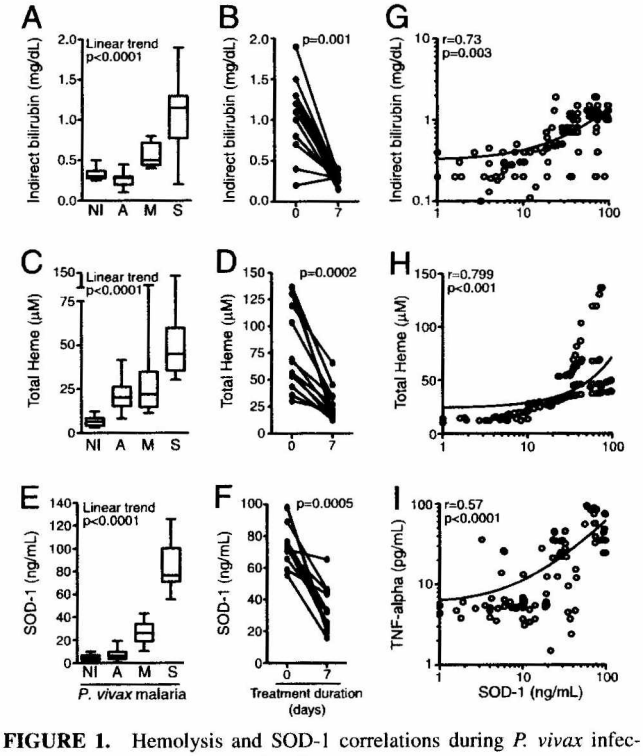

FIGURE 1. Hemolysis and SOD-1 correlations during *P. vivax* infection. Plasma concentrations of indirect bilirubin (*A*), total heme (*C*), and SOD-1 protein (*E*) were measured in noninfected individuals (NIs; n = 90) and in those with asymptomatic (A; n = 60), mild (M; n = 50), or severe (S; n = 19) *P. vivax* infection. Boxes represent medians and interquartile ranges, whereas whiskers represent maximum and minimum values. These data were analyzed using Kruskal-Wallis tests with linear trend posttests. In severe cases, indirect bilirubin (*B*), total heme (*D*), and SOD-1 (*F*) were also measured at the seventh day of the antimalarial treatment (Wilcoxon signed-rank paired tests were used to evaluate significant differences). *G–I* indicate correlations between the SOD-1 protein and indirect bilirubin, total heme, and TNF-α plasma concentrations, respectively, in malaria-infected patients (Spearman's test indicated significant correlations). The p values are shown for each graph.

and 48 h of stimulation with 3, 10, or 30 μM free heme using a LSR II cytometer (BD Biosciences).

Total heme measurement

Total heme in plasma samples was estimated by a colorimetric determination at 400 nm using the QuantiChrom Heme Assay Kit (BioAssay Systems, Hayward, CA), according to the manufacturer's protocol.

ELISA

SOD-1 protein levels and SOD activity were measured according to the manufacturer's protocols. PGE_2 levels were estimated in plasma samples or culture supernatants by enzyme-linked immunoassay, according to the manufacturer's instructions. After acidification to activate latent TGF-β followed by neutralization, total TGF-β1 was measured in the plasma or culture supernatants using ELISA, according to the manufacturer's instructions. In some experiments, the concentration of heme oxygenase-1 (HO-1) was estimated in cell lysates using the Human HO-1 ELISA kit from Assay Designs (Ann Arbor, MI), according to the manufacturer's protocol.

Small interfering RNA-mediated inhibition of SOD-1

SOD-1 and control small interfering RNAs (siRNAs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Transfection was performed according to the manufacturer's instructions.

Statistical analysis

Kruskal-Wallis tests with Dunn's test for multiple or selected pair comparisons or a linear trend analysis were used to compare SOD-1, total heme, indirect bilirubin, PGE_2, HO-1, and TGF-β1 levels. Mann-Whitney tests were used to verify differences between mild and severe malaria. The Wilcoxon matched pairs test was performed to estimate statistical significance before and during the antimalarial treatment. Correlations were performed using a Spearman's test. The p values ≤ 0.05 were considered to be statistically significant.

Results

Hemolysis and inflammation during vivax malaria

It is widely know that severe falciparum malaria is linked to oxidative stress (23), inflammation (2, 30, 31) and high levels of hemolysis (3). To determine whether the same is true for vivax malaria, we measured plasma levels of indirect bilirubin and SOD-1 protein in patients with different clinical presentations of *P. vivax* infection. Disease severity was directly linked to higher levels of hemolysis, as indicated by increased plasma concentrations of indirect bilirubin ($p < 0.0001$ for linear trend) (Fig. 1*A*, Table I). As expected, antimalarial drug treatment led to reduced concentrations of the indirect bilirubin in those individuals with severe infection ($p = 0.001$) (Fig. 1*B*). Total heme plasma concentrations presented strong linear trend according to the degree of the disease severity ($p < 0.0001$ for linear trend; Fig. 1*C*), with a significant reduction during treatment in those severely ill ($p = 0.0002$; Fig. 1*D*). Moreover, total heme levels positively correlated with plasma levels of indirect bilirubin (Spearman $r = 0.758$; $p = 0.001$). Plasma concentrations of SOD-1 protein also displayed a positive linear trend with the severity of the infection ($p < 0.0001$ for the linear trend) (Fig. 1*E*). Considering only the severe cases of the disease, the concentrations of this antioxidant enzyme were consistently reduced during the antimalarial therapy to amounts equivalent to those of the asymptomatic infection ($p = 0.0005$) (Fig. 1*F*). In addition, there was a positive correlation between the concentrations of SOD-1 protein and indirect bilirubin in all of the symptomatic patients infected with *P. vivax* (Spearman $r = 0.73$; $p = 0.003$) (Fig. 1*G*). SOD-1 was also positively correlated with plasma concentrations of total

Table I. *Baseline characteristics of the participants*

Variables	*P. vivax* Malaria			
	Noninfected ($n = 90$)	Asymptomatic ($n = 60$)	Mild ($n = 50$)	Severe ($n = 19$)
Male, no. (%)	39 (43.3)	30 (50.0)	22 (44.0)	10 (52.6)
Age (yr)[a]				
Median	38.0	42.0	33.0	22.0
Interquartile range	25.0–51.0	32.0–48.2	26.7–48.0	16.0–35.0
Previous malaria episodes[a]				
Median	14.0	16.0	8.0	3.5
Interquartile range	10.0–18.0	13.0–20.0	1.0–12	2.0–7.5
Years resident in the area[a]				
Median	11.4	12.5	7.4	3.0
Interquartile range	3.2–12.8	4.2–14.6	0.5–9.2	0.5–5.4
Parasitemia (parasites/μl)[a]				
Median	0	73[b]	4,798	49,358
Interquartile range	0	54.0–85.0	2,934–7,483	32,796–54,244
Hb (g/dl)[a]				
Median	13.2	11.5	8.9	6.4
Interquartile range	9.2–14.5	9.5–14.2	7.3–12.6	5.8–7.4
Serum creatinine (mg/dl)[a]				
Median	0.85	0.9	1.1	1.7
Interquartile range	0.7–1.2	0.7–1.2	0.7–1.3	1.42–2.45
ALT (U/l)[a]				
Median	42.35	40	58.3	238.4
Interquartile range	37.28–53.58	23.25–65.78	43.6–87.5	105.5–364.6
Total bilirubin (mg/dl)[a]				
Median	0.35	0.4	0.8	2.1
Interquartile range	0.3–0.4	0.3–0.62	0.7–2.05	1.15–3.1
Indirect bilirubin (mg/dl)[a]				
Median	0.3	0.28	0.5	1.1
Interquartile range	0.28–0.37	0.2–0.3	0.45–0.72	0.6–1.3

Ordinal variables were compared using the Kruskal Wallis test with Dunn's multiple comparisons. The prevalence of male gender was compared between the groups using χ^2 test.

[a]Differences were significant between groups ($p < 0.05$).

[b]Six of 60 individuals with asymptomatic *P. vivax* infection were negative for malaria infection by light microscopy but were positive for *P. vivax* infection by nested PCR.

ALT, alanine aminotransferase; CRP, C-reactive protein.

heme (Spearman $r = 0.799$; $p < 0.0001$) (Fig. 1*H*) and TNF-α (Spearman $r = 0.57$; $p < 0.0001$) (Fig. 1*I*).

We further assessed the plasma concentrations of PGE_2 and TGF-β, two anti-inflammatory molecules associated with *P. falciparum* infection (19, 22, 32, 33). Both PGE_2 and TGF-β plasma concentrations were elevated during *P. vivax* infection (Fig. 2*A*, 2*B*). Moreover, individuals with severe cases of the disease presented lower amounts of these mediators compared with those presenting mild infections (Fig. 2*A*, 2*B*). Both PGE_2/TNF-α (Fig. 2*C*) and TGF-β/TNF-α (Fig. 2*D*) ratios presented a decreased linear trend according to the disease severity.

Levels of PGE_2 and TGF-β increased during the antimalarial treatment ($p = 0.004$ and $p = 0.003$, respectively) (Fig. 2*E*, 2*F*), resulting in increased PGE_2/TNF-α and TGF-β/TNF-α ratios to levels similar to those of noncomplicated infections ($p = 0.01$ and $p = 0.003$, respectively) (Fig. 2*G*, 2*H*). Platelets are a rich source of latent TGF-β (34), and little is known about its ability to generate active TGF-β. In those individuals infected with *P. vivax*, the platelet count was lower than in those who are noninfected ($p = 0.027$) (Fig. 2*I*). In addition, severely ill patients presented lower platelet count compared with those with uncomplicated infection ($p = 0.042$) (Fig. 2*I*). SOD-1 is present in RBCs, and the elevated levels of this enzyme could be a result of the increased degree of hemolysis seen during malaria. As expected, individuals with severe disease also displayed lower RBC count than those with mild or asymptomatic malaria ($p = 0.038$) (Fig. 2*J*). Within the severely ill patients, plasma concentrations of PGE_2 were negatively correlated those of SOD-1 (Spearman $r = -0.68$, $p = 0.003$) (Fig. 2*I*), with similar patterns being observed with regard to TGF-β and SOD-1 (Spearman $r = -0.48$; $p = 0.051$) (Fig. 2*J*). This indicates that during vivax malaria, patients with severe disease present high hemolysis linked to higher SOD-1 levels. It also suggests that, compared with what is seen in uncomplicated infection, these events are correlated with impaired systemic release of both PGE_2 and TGF-β.

The interference of the anti-inflammatory responses can sustain proinflammatory responses in elevated activity, which can ultimately result in the systemic collapse observed in severe cases of malaria.

Heme triggers SOD-1 and impairs PGE_2 and TGF-β production in PBMC from healthy individuals

We hypothesized that free heme release from cell-free Hb acts in a pro-oxidant manner to induce the expression of SOD-1 favoring systemic inflammation. To test this hypothesis, we cultured fresh PBMCs from healthy volunteers residing in a nonendemic malaria region with increasing doses of free heme (3, 10, and 30 μM) and assessed the levels of SOD-1 protein, SOD activity, PGE_2, and TGF-β in the supernatants. Within the concentrations used in our experiments, heme was not very toxic for the cells, because the number of live cells did not significantly differ from unstimulated cells (Fig. 3*A*). Moreover, free heme induced the secretion of SOD-1 in the supernatants, an effect that was dose dependent ($p < 0.05$ at 24 and 48 h compared with unstimulated cells) (Fig. 3*B*). Following a similar trend, the SOD activity was also induced by heme ($p < 0.05$ at 6 and 48 h compared with unstimulated cells) (Fig. 3*C*). In addition, unstimulated cells presented an increased production of both PGE_2 (Fig. 3*D*) and TGF-β (Fig. 3*E*) over time, whereas those cultured with 30 μM heme did not ($p < 0.05$ at 48 h poststimulation). The next step was to verify whether this finding could be reproduced by a component of the heme molecule (LPS-free $FeSO_4$ or PPIX) or by an iron-carrier protein (LPS-free albumin or apotransferrin). After 48 h of stimulation, only heme was capable of increasing SOD-1 protein concentrations ($p < 0.05$ compared with unstimulated cells) (Fig. 4*A*) and reducing both PGE_2 and TGF-β release in the supernatants ($p < 0.05$ for each comparison with unstimulated cells) (Fig. 4*B*, 4*C*). Therefore, these outcomes

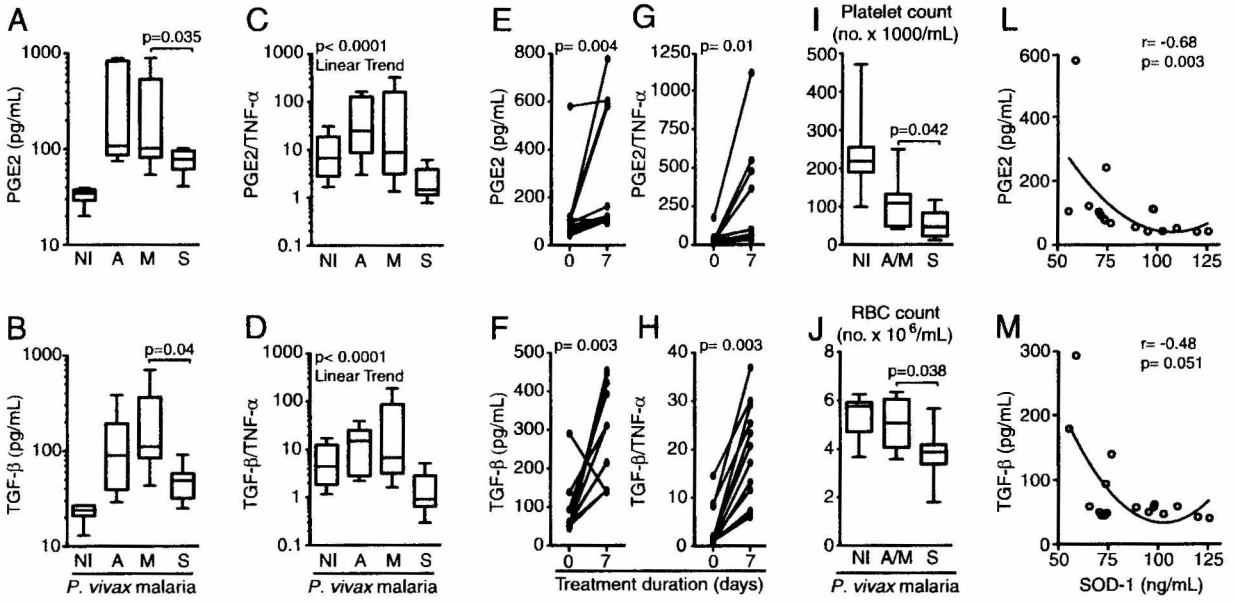

FIGURE 2. Unregulated anti-inflammatory responses during acute clinical attack of malaria. Plasma concentrations of PGE_2 (*A*) and TGF-β (*B*) were measured in uninfected individuals (NIs; $n = 90$) and in those with asymptomatic (A; $n = 60$), mild (M; $n = 50$), or severe (S; $n = 19$) *P. vivax* infection. *C* and *D* show PGE_2/TNF-α and TGF-β/TNF-α ratios, respectively. In severe cases, PGE_2 (*E*), TGF-β (*F*), PGE_2/TNF-α (*G*), and TGF-β/TNF-α (*H*) were also measured at the seventh day of the antimalarial treatment (Wilcoxon signed-rank paired tests were used to evaluate significant differences). Platelet counts (*I*) and RBC count (*J*) were estimated in the individuals. Boxes represent medians and interquartile ranges, and whiskers represent maximum and minimum values. Correlations between plasma concentrations of SOD-1 and PGE_2 (*L*) or TGF-β (*M*) were performed on patients with severe malaria at hospitalization using a Spearman's test (with a nonlinear curve fit). The *p* values are plotted in each graph. Data were analyzed using a Kruskal-Wallis test and a linear trend posttest. The *p* values are shown in each graph.

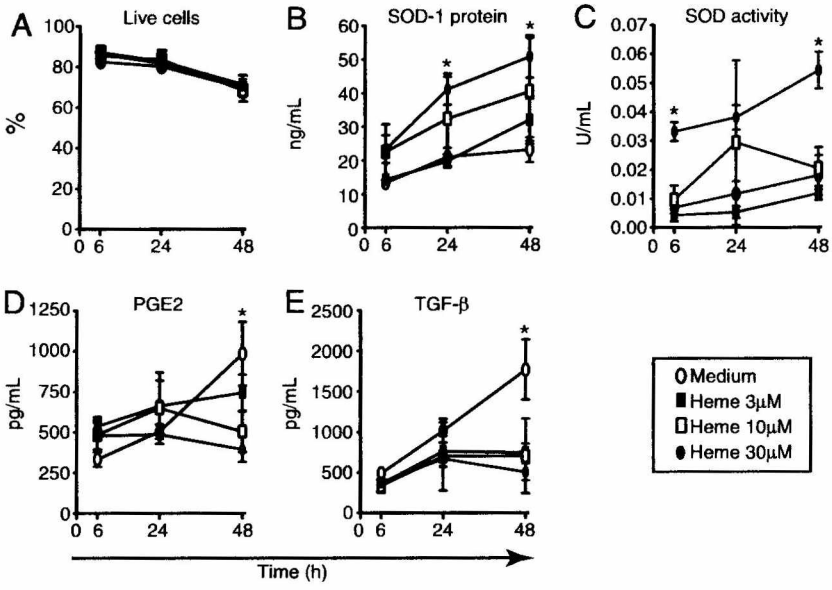

FIGURE 3. Heme triggers SOD-1 and impairs PGE_2 and TGF-β production. PBMCs (10^6/well) from seven healthy volunteers from a nonendemic malaria area were cultured with different doses of free heme (3, 10, and 30 μM). Percentage of live cells was estimated by counting cells unstained for both 7-AAD and Annexin V using flow cytometry (*A*). SOD-1 protein (*B*), SOD-1 activity (*C*), PGE_2 (*D*), and TGF-β (*E*) were measured in the supernatants at different times after stimulation (6, 24, and 48 h). Symbols represent mean, and whiskers represent SD. Data were analyzed using a Kruskal-Wallis test with Dunn's selected pairs (the conditions were compared with those cultured with the medium alone).

point to a possible intrinsic relationship between free heme, SOD-1, PGE_2, and TGF-β, as suggested by our results of human vivax malaria cases.

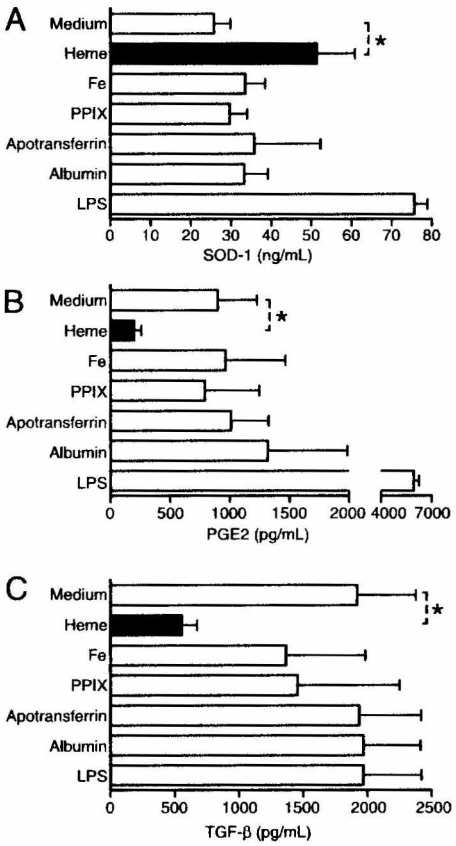

FIGURE 4. The reduction of PGE_2 and TGF-β is induced specifically by heme. PBMCs (10^6/well) from six healthy volunteers were stimulated with heme 30 μM or other stimuli such as $FeSO_4$ (30 μM), PPIX (50 μM), apotransferrin (100 μg/ml), albumin (100 μg/ml), or LPS (100 ng/ml) for 48 h, and SOD-1 protein (*A*), PGE_2 (*B*), and TGF-β (*C*) were measured in the culture supernatants. Bars and lines represent means and SD. Data were analyzed using a Kruskal-Wallis test with Dunn's selected pairs (the conditions were compared with those cultured with the medium alone). $*p < 0.05$. Other differences were considered to be significant and are described in the text.

The effect of Heme on PGE_2 and TGF-β production requires SOD-1

The idea that SOD-1 could hamper the regulatory responses to heme stimulation led us to actively interfere with SOD-1 activity and examine the resulting effects on PGE_2 and TGF-β. Interestingly, when heme-stimulated PBMCs from healthy volunteers were cultured in the presence of DETC, a cupper chelator that can inhibit SOD-1 activity (35), the inhibitory effect of heme on both PGE_2 and TGF-β release was reverted ($p = 0.01$ and $p = 0.04$, respectively, compared with cells stimulated with heme alone) (Fig. 5*A*, 5*B*). Moreover, when we treated the cells with NAC, a free radical scavenger, the concentrations of PGE_2 and TGF-β in the supernatants were reduced to a similar level as that observed with the use of heme ($p < 0.0001$ and $p = 0.004$, respectively, compared with unstimulated cells) (Fig. 5*A*, 5*B*). NAC was not capable of reducing the TGF-β concentrations in cells treated with heme plus DETC (Fig. 5*B*). In addition, when the cells were treated with exogenous catalase, the effect of heme on both PGE_2 and TGF-β was only partially reverted (data not shown). This suggests that SOD-1 might be a required source for H_2O_2 in this system and that the effect of heme on the production of PGE_2 and TGF-β is only partially mediated by the release of free radicals. Consequently, the activity of SOD-1 could play another role in this system. We then tried to test whether the heme effect on SOD-1 would be mediated by HO-1, an antioxidant enzyme that presents a major role in the pathogenesis of severe malaria (10, 36). As expected, heme induced high concentrations of HO-1 in PBMCs (Fig. 5*C*), an effect that was enhanced in the presence of CoPPIX, a HO-1 inductor, but nonaltered when cells were cocultured with SnPPIX, an inhibitor of the HO-1 activity (Fig. 5*C*). Nevertheless, neither CoPPIX nor SnPPIX interfered with SOD activity induced by heme in our model (Fig. 5*D*), suggesting that the heme effect on SOD is not directly mediated by HO-1.

To evaluate the direct role of SOD-1 on the reduction of PGE_2 and TGF-β mediated by heme, we successfully inhibited SOD-1 production using siRNA (Fig. 6*A*). siRNA-mediated SOD-1 inhibition before the addition of heme completely reverted the effect of heme on PGE_2 ($p < 0.0001$) (Fig. 6*B*) and TGF-β levels ($p < 0.0001$) (Fig. 6*C*).

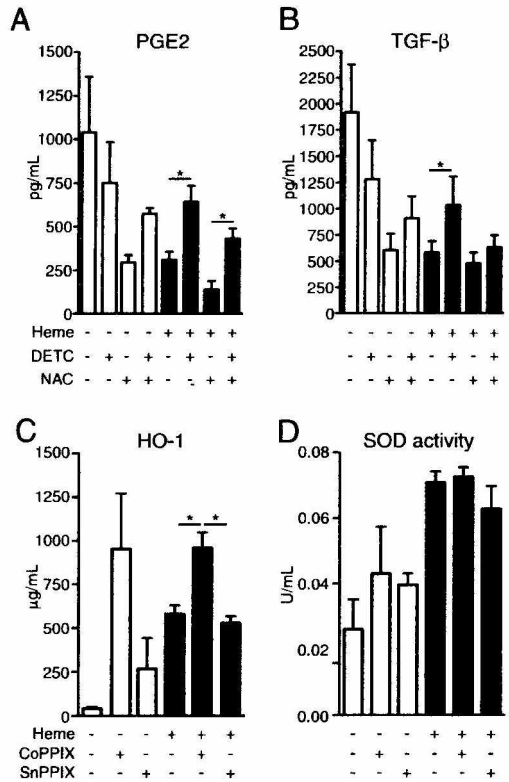

FIGURE 5. The role of SOD-1 on the release of PGE_2 and TGF-β. PBMCs (10^6/well) from six healthy volunteers were cultured in the presence of heme (30 μM) and/or DETC (2 mM) and/or NAC (20 mM) and/or CoPPIX (50 μM), and/or SnPPIX (50 μM) for 48 h as described in *Materials and Methods*. PGE_2 (*A*), TGF-β (*B*), and SOD activity (*D*) were measured in the supernatants, whereas HO-1 (*C*) protein levels were measured in cell extracts. Bars and lines represent means and SD. A Kruskal-Wallis test with Dunn's multiple comparisons or selected pairs was used to evaluate statistical significance. $*p < 0.05$. Other differences were considered to be significant and are described in the text.

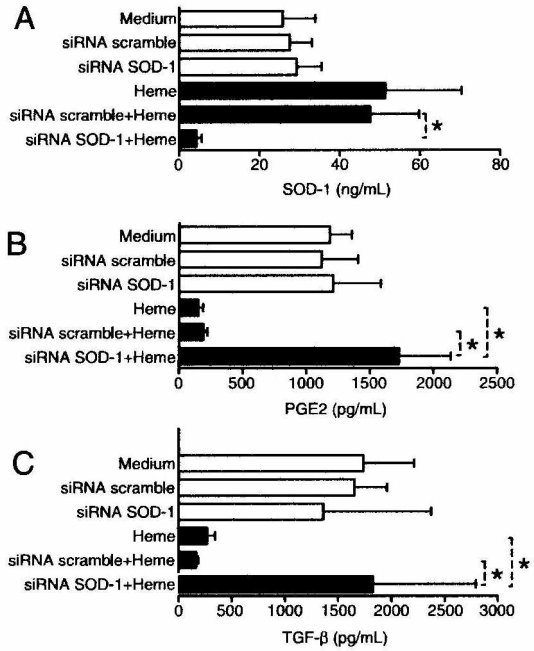

FIGURE 6. The effect of Heme on PGE_2 and TGF-β production requires SOD-1. PBMCs from six healthy volunteers (10^6/well) were cultured with heme (30μM) in the presence of siRNA for SOD-1 as described in *Materials and Methods*. After 48 h of stimulation, SOD-1 protein (*A*), PGE_2 (*B*), or TGF-β (*C*) was measured in the supernatants. Bars and lines represent means and SD. Data were analyzed using a Kruskal-Wallis test with Dunn's multiple comparisons. $*p < 0.01$.

The role of CD14 on the heme-impaired production of PGE_2 and TGF-β

Previous studies demonstrated that heme induces TNF-α production by human and murine macrophages through the activation of TLR4 and CD14 (37). In an attempt to verify whether the effect of heme on SOD-1 as well as on the release of PGE_2 and TGF-β would be mediated by CD14, we incubated fresh PBMCs from normal volunteers with anti-CD14 for 1 h prior to stimulation with heme. As expected, the neutralization of CD14 adequately reversed the effect of LPS on PGE_2 release but not the effect of TGF-β ($p < 0.0001$ and $p = 1.0$; comparing LPS plus anti-CD14 with LPS alone) (Fig. 7). In addition, the blockage of CD14 reduced SOD-1 ($p < 0.05$) (Fig. 7*A*) and increased the concentrations of PGE_2 in supernatants from cells stimulated with heme ($p < 0.05$) (Fig. 7*B*) but did not alter the levels of TGF-β ($p = 1.0$) (Fig. 7*C*). Thus, it seems that the direct effect of heme on the suppression of PGE_2 involves at least in part the binding to CD14.

Discussion

In malaria, as in diseases with release of Hb from RBCs, there is uncontrolled inflammatory imbalance and intense oxidative stress (2, 38–40). In this paper, we report that patients with severe *P. vivax* infection, similar to individuals and mice with falciparum malaria (22, 33, 41), displayed reduced plasma levels of PGE_2 and TGF-β than those with mild infections, with this reduction being inversely proportional to the bilirubin, total heme, and SOD-1 levels. Despite these similarities, one important difference can be noted between the current study and those made in falciparum malaria, which is the fact that plasma PGE_2 (22) and TGF-β (33) are lower in mild and severe falciparum malaria than in normal controls. In our study, independently of the disease severity, *P. vivax* infection was linked to an increase in the plasma concentrations of both PGE_2 and TGF-β. These data can indicate an important difference between vivax and falcipaum malaria. In this scenario, patients with *P. vivax* infection present a significant augmentation of circulating T regulatory cells producing TGF-β, and this production directly relates to the parasite load (42). This difference and other possible disparities in the degree of hemolysis and thrombocytopenia between patients with falciparum and vivax malaria need further investigation.

The association between systemic concentrations of SOD-1 and indirect bilirubin in the individuals presenting with malaria was not surprising as SOD-1 is present in RBCs (43, 44) and the severely ill individuals from our study presented lower RBC count than those with uncomplicated infection. For this reason, we tested whether human PBMCs were able to release SOD-1 in the presence of heme. Interestingly, free heme reduced both PGE_2 and TGF-β via SOD-1 production and activity in human PBMCs. These events may influence the capacity of critically ill patients to produce adequate inflammatory responses.

The possible role of SOD-1 in the pathogenesis of human malaria has only superficially been addressed before, with some degree of speculation. Patients with acute noncomplicated *P. falciparum* or *P. vivax* malaria have a lower catalase activity than noninfected individuals but a higher SOD activity (27). Reduced catalase activity, together with increased SOD activity, may result in the accumulation of H_2O_2. In the presence of divalent metals such as the iron contained within the protoporphyrin ring of heme, H_2O_2 is

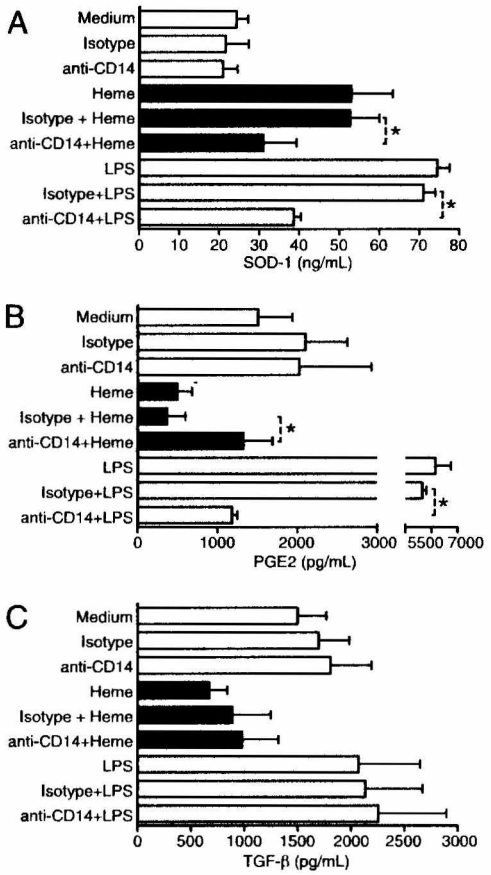

FIGURE 7. The role of CD14 on the heme-mediated effects. PBMCs from six healthy volunteers (10^6/well) were cultured with heme (30 μM) in the presence of anti-CD14 or a control isotype (10 μg/ml) as described in *Materials and Methods*. After 48 h of stimulation, SOD-1 proteins (*A*), PGE_2 (*B*), and TGF-β (*C*) were measured in the supernatants. Bars and lines represent means and SD. Data were analyzed using a Kruskal-Wallis test with Dunn's multiple comparisons. $*p < 0.05$.

rapidly converted into hydroxyl radicals, which promote tissue damage in a manner that compromises the viability of a *Plasmodium*-infected host (11). Our study showed that the direct association between hemolytic activity and SOD-1 during malaria could cause defects in the regulatory responses that could favor disease severity.

Severe malaria syndromes are heterogeneous conditions resulting from complicated infections (5). The characteristics of severe vivax malaria are controversial, because the specific pathogenesis of the clinical complications is still poorly understood. In the present paper, we used the previously indicated characteristics to define severe vivax cases (8), which are adapted from the criteria for *P. falciparum* malaria severity. Despite the heterogeneity of malaria clinical presentations, elevated hemolysis was a major symptom presented in all severe cases, documented in this paper by the measurement of indirect bilirubin and also by the estimation of total heme plasma concentrations. In addition, both heme and indirect bilirubin are elevated in experimental cerebral malaria (10), in noncerebral forms of severe malaria (11), as well as in other hemolytic disorders (45). Moreover, there is experimental evidence to suggest that heme release from Hb contributes in a critical manner to malaria-related immunopathology (10, 11, 36). During *Plasmodium* infection, the parasites metabolize free heme molecules into hemozoin. It is well known that *P. falciparum*-derived hemozoin exerts many different pathological effects on the host, such as inducing inflammation (46) and reducing cyclooxygenase-2–mediated PGE_2 production (19). The results presented in this paper suggest that general priming of inflammation could be mediated by heme itself, which causes ROS and TNF-α production in macrophages (37) and hampers PGE_2 and TGF-β secretion, similar to hemozoin. Therefore, patients with severe forms of *P. vivax* or *P. falciparum* malaria who present elevated hemolysis display two major inflammatory stimuli, free heme and hemozoin. We did not analyze possible effects of heme on the adaptive immune responses, as has been described for hemozoin (47). It is plausible to expect some degree of influence, as heme induces important oxidative stress.

To our knowledge, this is the first paper on the direct effect of heme on SOD-1, PGE_2, and TGF-β secretion by human cells. Most of the data regarding the effect of heme on oxidative stress involves the anti-oxidant enzyme HO-1 (12, 48). Interestingly, some antioxidant mechanisms of HO-1 on vascular cells require the production of extracellular SOD-1 (49, 50). In the current study, neither the HO-1 inductor CoPPIX nor the inhibitor of HO-1 activity SnPPXI interfered with SOD activity, suggesting that the heme effect on SOD is not directly mediated by HO-1. Aside from playing a protective role during oxidative stress, SOD-1 activity has been linked to several inflammatory diseases and infections, including malaria (26, 27, 51–53).

In the current study, we demonstrated that heme triggers SOD-1 release, at least in part, by binding to CD14. Heme can activate TLR4/CD14 and induce TNF-α production macrophages (37). However, ROS production induced by heme is not dependent on TLR4 (37). Our findings suggest that the consequence of ROS production is not the single mechanism responsible for the negative regulation of PGE_2 and TGF-β by heme, because this effect was reduced when we neutralized the SOD-1 activity using DETC. Thus, SOD-1 may trigger an effect that is still unknown and that could synergize with the ROS produced. This hypothesis was confirmed by the reversal of the heme effect on PGE_2 and TGF-β when PBMCs were cultured in the presence of SOD siRNA. Interestingly, LPS, a CD14 agonist, is a potent inductor of PGE_2 (54), and it induces TNF-α production via SOD-1 release as a consequence of ERK-1 phosphorylation (24). In our study, blocking CD14 led to a reversal of the heme effect on PGE_2 secretion but had no effect on TGF-β levels. Although heme and LPS bind to the same receptor, they have opposite effects on the production of PGE_2. Investigating the divergent effects of heme and LPS is beyond the scope of this paper. Unexpectedly, a CD14 blockage did not influence the effect of heme on TGF-β, indicating a diverse mechanism in its relationship with PGE_2. Heme-induced TGF-β reduction may involve ROS production rather than binding to a specific receptor. Recent studies have shown that superoxide anions increase the release of TGF-β1 and collagen from human lung fibroblasts (55). In our study, enhanced SOD-1 secretion could scavenge superoxide radicals, negatively impacting the production of TGF-β. The release of free heme during hemolysis could exert effects that are dependent on ROS generation and are also dependent on CD14 binding. SOD-1 appears to be involved in both situations.

In many infectious conditions, uncontrolled activation of the inflammatory responses is strongly associated with severe outcomes. Conversely, indiscriminate immunosuppression is linked to disseminated infection and death. Therefore, an adequate modulation of the immune responses can minimize immunopathology while limiting the infectious agent. The elevation of proinflammatory mediators is usually accompanied by the increase of anti-inflammatory factors. This balance determines the outcomes of the infections. With regard to malaria, high ratios of proinflammatory to anti-inflammatory cytokines are associated with increased symptoms (56) and disease severity (8). Intriguingly, heme hampers regulatory responses by reducing TGF-β and PGE_2 while also

inducing inflammation, which favors uncontrolled inflammation. To better understand the specific mechanism underlying this finding, a detailed description of the intracellular signaling pathways involved is urgently needed.

In a broad context, the inhibition of SOD-1 could engage the host's immune system and reinforce the regulatory responses that could ultimately diminish the severity of the disease. The SOD-1 inhibitor DETC has also been used in vivo as an adjuvant of the immune system, delaying the disease progression in HIV-infected patients (57). However, this approach would need to be cautiously evaluated before being used in humans because the consequences of interfering with highly complex systems are unknown. The insights into the pathogenesis of severe malaria reported here may present new and interesting approaches for the management of human diseases where hemolysis is an important element.

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Disclosures

The authors have no financial conflicts of interest.

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6. DISCUSSÃO

Esta tese traz uma coletânea de manuscritos que abordam diversos aspectos da malária no estado de Rondônia, Brasil. Seis trabalhos apresentam resultados que em conjunto traçam considerações importantes para o direcionamento de futuras intervenções na área.

Antes de conseguir investigar os fatores imunológicos associados à infecção assintomática ou grave, tornou-se imperativo avaliar corretamente o diagnóstico da infecção. Para tanto, diversos testes diagnósticos foram comparados no primeiro manuscrito. Através de busca ativa e passiva de casos foi possível rastrear indivíduos com diferentes probabilidade pré-teste de ter malária. No nosso estudo, o método do nested-PCR mostrou-se o mais eficaz na identificação dos casos sintomáticos e assintomáticos, independente da parasitemia. Este achado já foi observado em estudos anteriores (Zalis *et al.*, 1996; Coleman *et al.*, 2006). Quanto à identificação de casos com baixa parasitemia, o nosso estudo mostrou o achado surpreendente de que o teste rápido avaliado (Optimal-IT) apresentou maior sensibilidade do que a microscopia. Poucos estudos anteriores exploraram o uso de testes rápidos na região amazônica (Arcanjo *et al.*, 2007; Metzger *et al.*, 2008). Em outras regiões, os testes rápidos apresentam eficácia semelhante ao da microscopia (Iqbal *et al.*, 2002; Pattanasin *et al.*, 2003; Ashley *et al.*, 2009; Valea *et al.*, 2009). Outro resultado que atrai a atenção é o fato de que mesmo o melhor método diagnóstico (nested PCR) identificou o plasmódio em apenas 55,6% dos indivíduos que apresentavam sintomas compatíveis com malária e que procuraram o centro diagnóstico da FUNASA. Os indivíduos com outras doenças procuraram a exclusão da malária antes de buscar atendimento nas

clínicas de atendimento primário, provavelmente devido à alta prevalência desta infecção no município. Este costume pode levar a um atraso no diagnóstico correto e, em casos graves, pode comprometer um manejo adequado precoce, afetando diretamente o prognóstico e os custos dos cuidados de saúde. Os dados deste trabalho também mostram que nessa região há um baixo desempenho do exame microscópico para o diagnóstico de malária. Isto pode ser conseqüência de uma deficiência no treinamento continuado dos técnicos microscopistas da FUNASA, ou mesmo de investimento em reagentes ou novos microscópios. Embora importante, esta questão não foi aprofundada no trabalho. Assim, foi estabelecido que o nested PCR seria o exame utilizado nas nossas futuras abordagens para o diagnóstico da malária.

Nós desenvolvemos um programa de computador que utilizou redes neurais artificiais para o diagnóstico de casos com malária assintomática. Em uma investigação piloto, nossos colaboradores encontraram que esta metodologia evidenciava associações mais fortes do que a análise tradicional utilizando a estatística tradicional (regressão logística) e a estatística bayesiana (Duarte *et al.*, dados não publicados). O programa baseado em redes neurais apresentou desempenho inferior ao da microscopia, quando utilizou na sua rede de neurônios apenas variáveis epidemiológicas. Este achado sugere que apesar de apresentarem fortes associações com a infecção assintomática, as variáveis epidemiológicas não são capazes de gerar boa predição diagnóstica. O nosso estudo evidenciou que o perfil imunológico parece ser importante nesse processo, no momento em que a rede neural apresentou um sucesso muito maior na predição quando utilizou dados referentes aos níveis séricos de IL-10 e IFN-gama. Neste e nos outros estudos, nós usamos estas duas citocinas porque a razão entre elas tem sido utilizada para correlacionar o balanço das respostas

imunológicas com a gravidade da doença (Metenou *et al.*, 2009). Assim, o conhecimento sobre o perfil imunológico parece ser fundamental para a ocorrência da imunidade clínica na malária.

O conhecimento sobre os determinantes da imunidade clínica contra a malária ainda é escasso. A maioria dos trabalhos descreve fatores associados à malária assintomática por *P. falciparum*. A malária causada pelo *P. vivax* foi por muito tempo negligenciada nesse sentido, devido a sua reduzida letalidade. A maioria dos estudos relaciona fatores epidemiológicos com a ocorrência da malária assintomática. Nós consideramos válido investigar a relação entre a exposição a picadas do vetor *An. darlingi* nesse contexto. Em outras doenças infecciosas, como a leishmaniose visceral humana, a exposição aos vetores pode ser avaliada nas áreas endêmicas pela positividade da reação sorológica contra a saliva do vetor (Barral *et al.*, 2000) e isto se correlaciona temporalmente com o desenvolvimento de resposta imunológica contra o parasita (Gomes *et al.*, 2002). Seguindo linha semelhante, estudos experimentais mostraram que camundongos expostos repetidamente à picada de mosquitos *An. stephensi* não infectados apresentam um aumento das respostas Th1 que limita a infecção por *P. yoelii* (Donovan *et al.*, 2007). Além disso, em comunidades africanas, a quantificação de anticorpos contra saliva de mosquitos vetores pode ser usada para estimar o risco potencial de malária (Remoue *et al.*, 2006; Orlandi-Pradines *et al.*, 2007). No nosso segundo manuscrito nós evidenciamos a quantificação de anticorpos anti-saliva do *An. darlingi* como marcador de imunidade clínica na malária vivax. Nós escolhemos focar na infecção por *P. vivax* porque este parasita é o mais prevalente na região estudada (80% dos casos). Indivíduos com maiores títulos de anticorpos anti-saliva apresentaram menor razão IFN-gama/IL10, sugerindo que a

associação entre a resposta humoral e a proteção clínica envolve um balanço adequado de citocinas. Este foi o primeiro estudo na Amazônia brasileira a testar essa associação. Apesar de intrigante, este trabalho foi uma investigação inicial que utilizou uma amostra pequena para testes de predição, em razão da disponibilidade limitada de saliva dos insetos. Além disso, o estudo não mensurou anticorpos contra o *P. vivax*. Pode ser que os anticorpos contra a saliva do vetor somente reflitam a resposta humoral contra o parasita, e que podem não influir diretamente na indução da imunidade clínica. Embora a função específica nesse processo não tenha sido identificada, a resposta humoral contra componente salivares do *An. darlingi* mostra-se como um poderoso marcador de casos de malária vivax assintomática.

Outro fator que certamente afeta a apresentação clínica da malária é a presença de co-infecções. As áreas endêmicas para malária, incluindo a Amazônia brasileira, apresentam geralmente alta endemicidade para várias outras doenças infecciosas, como dengue, helmintíases, e hepatites virais (Da Silva Jr, 2006). Nesse contexto, o estado de Rondônia apresenta uma das maiores prevalências de malária no Brasil, e também apresenta a maior prevalência de hepatite viral B (Da Silva Jr, 2006). Estudos anteriores nessa região sugerem que a prevalência de malária assintomática é alta e pode ser até cinco vezes maior do que os casos sintomáticos (Alves *et al.*, 2002). Por esse motivo nós decidimos avaliar o impacto da hepatite viral B na apresentação clínica da malária. Estudos prévios investigaram essa relação no estado do Amazonas mas as evidências foram frágeis e a metodologia utilizada para a identificações dos casos foi primária, utilizando apenas sorologia (Souto *et al.*, 2001; Braga *et al.*, 2005; Braga *et al.*, 2006). O nosso achado de que a infecção pelo vírus

da hepatite B (VHB) se correlaciona fortemente com a ocorrência da malária assintomática vai contra dois trabalhos prévios em outras regiões (Thursz *et al.*, 1995; Barcus *et al.*, 2002). Tais trabalhos avaliaram apenas pacientes admitidos em hospital e/ou apresentando malária grave. O nosso estudo realizou busca ativa e passiva de casos e investigou em sua maioria casos de malária não complicada. Na nossa casuística, os pacientes com malária grave foram negativos para a hepatite viral B e muitas outras co-infecções. Além disso, o estudo utilizou diagnóstico robusto utilizando técnicas sorológicas e moleculares. Outra informação válida em nosso estudo foi que os pacientes co-infectados apresentam valores da razão IFN-gama/IL-10 reduzidos quando comparados com os indivíduos portadores somente com a hepatite B ou malária. De uma maneira intrigante, os indivíduos que possuíam marcadores de exposição prévia ao VHB também apresentaram resultados semelhantes. Os indivíduos co-infectados não apresentaram elevação dos parâmetros laboratoriais de disfunção orgânica, como bilirrubinas, proteína C reativa, fibrinogênio, transaminases hepáticas e creatinina sérica. Isso sugere que um provável mecanismo que pode favorecer a malária assintomática nos indivíduos co-infectados seria a interferência no balanço de citocinas e da atividade inflamatória. As análises mostradas no manuscrito foram feitas considerando tanto as infecções por *P. vivax* e *P. falciparum*, e quando foram considerados apenas os casos de malária vivax, as mesmas associações se mantiveram. Portanto, apesar da malárias causada por *P. vivax* e *P. falciparum* serem consideradas diferentes do ponto de vista imunopatológico, existem fatores em comum que se relacionam com a magnitude da apresentação clínica da doença.

Outro achado importante do estudo da co-infecção foi o fato dos pacientes co-infectados apresentarem maiores viremias do que os infectados com o VHB somente. Estudos prévios evidenciaram resultados semelhantes em um menor número de pacientes com o *P. falciparum* (Brown *et al.*, 1992). Assim, os indivíduos co-infectados apresentam maior chance de permanecerem assintomáticos e ao mesmo tempo sustentam maior viremia, o que pode favorecer a cronificação do vírus. O nosso estudo não fez seguimento dos pacientes co-infectados e portanto não pode inferir mais do que a associação referida. Entretanto, os resultados podem em última análise explicar porque as duas infecções são altamente prevalentes em muitas regiões o mundo. Como o objetivo primário do trabalho era avaliar a co-infecção natural, nós não avaliamos o papel da vacinação contra a hepatite viral B nesse processo. Isso será realizado em futuras abordagens. Nós também não avaliamos a razão pela qual os indivíduos com marcadores de infecção pregressa pelo VHB apresentaram também maior chance de terem malária assintomática. Os indivíduos que cronificam a hepatite viral B geralmente apresentam perfil inflamatório distinto daqueles que se curam (Boonstra *et al.*, 2008; Liaw, 2009; Wang e Zhang, 2009). É sugerido que os indivíduos que cronificam a infecção pelo VHB não conseguem estruturar uma forte resposta Th1 (Boonstra *et al.*, 2008; Wang e Zhang, 2009). Nós mostramos que esses indivíduos apresentam altos níveis séricos de IFN-gama. Pode ser que apesar de insuficiente para eliminar a infecção viral, a resposta mediada pelo IFN-gama pode ser útil no controle da infecção pelo plasmódio. É importante ressaltar que este trabalho investigou a hepatite viral B no contexto da malária. Não foi objetivo do trabalho estudar profundamente a hepatite viral. Nos indivíduos co-infectados que apresentavam-se sintomáticos, por exemplo, não sabemos se os sintomas foram causados pela malária ou hepatite viral. Todos os indivíduos positivos para hepatite

viral foram aconselhados a procurarem um serviço de referência do estado (CEMETRON).

Os três primeiros estudos que compõem a tese investigaram diferentes aspectos da malária assintomática. Igualmente importante é a identificação de marcadores confiáveis de malária grave, a fim de melhorar a assistência de saúde e reduzir a carga de doença. Quando comparado às outras espécies, o *P. falciparum* causa maior morbimortalidade, além de apresentar crescente resistência à cloroquina e outras drogas, já detectada na maioria das zonas endêmicas conhecidas (Talisuna *et al.*, 2007). A doença causada pela infecção pelo *P. vivax*, por outro lado, por muito tempo foi considerada uma doença benigna, com raras complicações (Mueller *et al.*, 2009). Apesar disso, há um aumento significativo da resistência do *P. vivax* à cloroquina (Baird, 2004; De Santana Filho *et al.*, 2007). Diante do fato de termos uma amostra maior de pacientes infectados pelo *P. vivax*, e da aparente negligência dada à malária vivax, nós decidimos focar as investigações nas infecções causadas por este parasita. Historicamente, os relatos de casos de malária complicada causada pelo *P. vivax* foram raros (Price *et al.*, 2009). Evidências recentes no entanto têm reforçado a associação entre a malária vivax, complicações graves e morte em outras áreas endêmicas (Barcus *et al.*, 2007; Genton *et al.*, 2008; Tjitra *et al.*, 2008). As complicações graves associadas à malária vivax também foram relatadas na região amazônica (Makkar *et al.*, 2002; Daniel-Ribeiro *et al.*, 2008). Uma análise superficial sobre os dados do ministério da saúde sobre a taxa de hospitalização por malária na região amazônica evidencia que a infecção pelo *P. vivax* apresenta índice semelhante à infecção pelo *P. falciparum* (Brasil, 2008). Estes mesmos dados revelam um alto numero de casos de malária nos quais não foi possível identificar a espécie o

plasmódio (Brasil, 2008). Considerando que o diagnóstico da malária vivax pode estar sendo subestimado, a morbidade pode ser muito maior do que a relatada oficialmente. Essa idéia é reforçada pelo fato de que durante um estudo no município de Buritis nós identificamos 19 casos de pacientes admitidos com alta parasitemia por *P. vivax* e com sintomas diversos de doença grave. A triagem dos casos foi consistente e a única espécie de plasmódio identificada pelos teste moleculares foi o *P. vivax*. Além disso, as principais manifestações clínicas apresentadas nos casos estão de acordo com relatos prévios no contexto do *P. vivax* (Price *et al.*, 2007). Uma série de possibilidades de co-infecção foram excluídas pelos médicos assistencialistas do hospital municipal e ainda pelos nossos testes sorológicos. Apesar disso, é possível que estes pacientes tenham infecções outras que não foram rastreadas. Mesmo diante desta limitação, se compararmos aos outros estudos da área, nosso trabalho apresenta uma triagem satisfatória dos casos. Além disso, vale ressaltar que não há critérios padronizados para o diagnóstico da malária vivax grave. Os critérios utilizados nesta série de trabalhos foram previamente padronizados para a infecção pelo *P. falciparum* (WHO, 2000). Vários trabalhos que estudaram a malária vivax grave usaram metodologia semelhante (Barcus *et al.*, 2007; Genton *et al.*, 2008; Tjitra *et al.*, 2008). No nosso trabalho, os indivíduos com doença grave apresentaram maiores níveis de parasitemia, TNF-alfa e IFN-gama, além de todos os parâmetros laboratoriais de lesão orgânica, do que os indivíduos sem complicações. Isso sugere que, assim como na infecção pelo *P. falciparum*, a malária vivax grave também está associada a ativação inadequada de cascatas inflamatórias sistêmicas. Curiosamente, em outros estudos a gravidade da malária vivax não está fortemente associada a altas parasitemias (Barcus *et al.*, 2007; Genton *et al.*, 2008; Tjitra *et al.*, 2008). Além disso, seis dos 19 pacientes morreram poucos dias depois da hospitalização, mostrando uma alta mortalidade. Os

indivíduos que morreram podem ter procurado a assistência médica tardiamente ou ainda podem ter tido resistência à cloroquina, mas esses dados não foram coletados para os seis pacientes. Os outros 13 pacientes não apresentaram resistência terapêutica e, apesar de terem sido admitidos com doença muito grave, apresentaram cura clínica. Pode ser que uma variante de *P. vivax* esteja associado a estes casos. Outro achado foi a reduzida concentração de IL-10 nos casos graves. Diferentes estudos mostraram que a IL-10 pode estar elevada nos casos graves de malária por *P. vivax* ou *P. falciparum* (Ageely *et al.*, 2008; Jain *et al.*, 2009), e que polimorfismos associados a IL-10 se associa com a doença grave causada pelo *P. falciparum* (Ouma *et al.*, 2008). A variabilidade genética do parasita e a influência de polimorfismos não foi investigada neste série de trabalhos. O quarto manuscrito foi portanto somente um primeiro passo na descrição inflamatória e imunológica da malária vivax grave e no entendimento de potenciais preditores desta condição.

Recentemente, tem sido uma prioridade do nosso laboratório o estudo de biomarcadores envolvidos na resposta inflamatória. Neste panorama, além do análise de perfis de citocinas, a investigação do estresse oxidativo aponta como uma promissora abordagem. Vários são os estudos em andamento do nosso grupo que tratam de estudar o perfil do metabolismo oxidativo no desfecho de infecções. Durante a infecção pelo plasmódio, um intenso estresse oxidativo já foi descrito, com produção de vários radicais livres, principalmente os ânions superóxido (Delmas-Beauvieux *et al.*, 1995). A enzima superóxido dismutase-1 (SOD-1) é importante a eliminação dos íons superóxidos (Dive *et al.*, 2003) e parece estar elevada durante a malária experimental (Golenser *et al.*, 1998) e humana (Pabon *et al.*, 2003). Como uma abordagem inicial para testar se essa enzima serviria como marcador de malária

vivax grave, nós comparamos o poder de predicção com o TNF-alfa, citocina que é associada aos paroxismos maláricos (Karunaweera *et al.*, 2003) e também é considerada marcador de gravidade na malária (Kern *et al.*, 1989). A enzima anti-oxidante apresentou-se como ou preditor de casos graves mais poderoso do que o TNF-alfa. Vale ressaltar que este estudo usou uma pequena amostra e que esses dados deverão ser validados em populações maiores, em estudos de seguimento. Além disso, a aplicabilidade clínica do uso da sorologia para SOD-1 ainda é incerta, pois não foi realizada análise de custo. Mais do que indicar um novo biomarcador, este trabalho nos evidenciou a possibilidade da SOD-1 estar desempenhando papel importante na imunopatogênese da doença, fato que foi explorado no último manuscrito da série. Até o momento, não tínhamos idéia se as concentrações sistêmicas elevadas da SOD-1 estavam contribuindo para o desfecho grave, como sugerido por estudos experimentais (Golenser *et al.*, 1998), ou somente representaria uma resposta do hospedeiro para evitar mais dano tecidual e restabelecer a homeostasia.

O próximo passo foi então avaliar se a SOD-1 tem papel direto na malária vivax grave. Além de ser relacionada com o inflamação e seqüestro de parasitas na periferia (Clark *et al.*, 2004; Schofield, 2007), a gravidade da malária causada pelo *P. falciparum* associa-se com uma hemólise mais intensa (Ferreira *et al.*, 2008). Como um mecanismo de proteção contra os efeitos tóxicos da hemoglobina livre, o plasmódio metaboliza moléculas de heme livre em cristais de hemozoína. Ao mesmo tempo que protege o parasita do dano oxidativo, a hemozoína causa alterações no sistema imunológico que resulta na inadequação das respostas anti-inflamatórias (Perkins *et al.*, 2003; Coban *et al.*, 2005; Keller *et al.*, 2006; Awandare *et al.*, 2007).

Nossos estudos com pacientes portadores de malária vivax grave evidenciaram elevados níveis de bilirrubina total, bilirrubina direta e bilirrubina indireta, sugerindo que esses pacientes também apresentam hemólise intravascular aumentada. Além disso, os pacientes com doença grave apresentaram níveis sistêmicos de prostraglandina E2 (PGE2) e TGF-beta reduzidos em comparação aqueles com doença não complicada, de maneira semelhante à infecção pelo *P. falciparum* (Keller *et al.*, 2006; Awandare *et al.*, 2007). Nós testamos se o heme livre estaria desempenhado papel semelhante à hemozoína no sistema imune do hospedeiro. Nosso trabalho mostrou que o heme livre influencia a redução de PGE2 e TGF-beta por células mononucleares do sangue periférico de doadores saudáveis e ainda que este efeito é mediado pela enzima SOD-1. O mecanismo específico que relaciona diretamente o heme livre a produção da SOD-1 e a redução de TGF-beta e PGE2 foi parcialmente explorada no nosso trabalho. O CD14 e o receptor do tipo toll 4 (TLR4) já foram descritos como ligantes do heme livre e inclusive foram relacionados a alguns dos seus efeitos, como migração celular e produção de radicais livres (Pacheco *et al.*, 2002; Figueiredo *et al.*, 2007). No nosso estudo, o efeito do heme sobre a SOD-1 e também sobre a produção de PGE2 envolveu parcialmente a sua ligação ao CD14. Entretanto, via relacionada à na produção de TGF-beta não foi esclarecida com as tentativas empregadas neste estudo, mas há indícios de que pode estar sendo regulada por um mecanismo que envolve produção de radicais livres (Qi *et al.*, 2009).

A caracterização das peculiaridades das respostas imunológicas relacionadas às diversas apresentações clinicas da malária é de fundamental importância para o direcionamento de futuras intervenções profiláticas e terapêuticas. Para que esta

caracterização seja possível, o diagnóstico preciso das diversas formas clínicas é necessário. Neste quesito, a série de estudos desta tese iniciou uma abordagem promissora na identificações de fatores relacionados tanto à imunidade clínica na quanto na gravidade, principalmente no escopo das infecções pelo *P. vivax*. Uma atenção especial foi dada à malária vivax devido a sua importância epidemiológica no Brasil e a aparente negligência com que vem sendo tratada por muitos malariologistas. Após estabelecimento de uma metodologia robusta para o rastreamento de casos, diversos candidatos a novos biomarcadores foram testados em estudos iniciais que servirão de base para futuras investigações. Além disso, alguns estudos apresentados aqui foram além e indicaram possíveis papéis desses biomarcadores na imunopatogênese das doenças. Assim, esta tese contribui em diversos aspectos para o entendimento da malária no Brasil.

7. CONCLUSÕES

Os achados dos trabalhos que compõem a tese levam as seguintes conclusões:

- A técnica do *nested PCR* apresenta-se como a mais eficiente para o diagnóstico da malária sintomática e assintomática, mas seu custo ainda torna o seu uso restrito na rotina dos postos diagnósticos;
- O uso racional do teste rápido *Optimal-IT* para o diagnóstico da malária pode ser promissor em áreas em que existe dificuldade de treinamento continuado dos recursos humanos da FUNASA;
- Apesar do baixo desempenho diagnóstico do teste baseado em redes neurais artificiais usando somente dados epidemiológicos, uma abordagem baseada em metodologia semelhante pode ser viável futuramente em casos onde métodos mais simples para discriminar indivíduos abaixo e acima de um limiar dos níveis de citocinas estejam disponíveis. Investimentos no software de redes neurais favorecerão o desenvolvimento de uma poderosa ferramenta diagnóstica ao SUS;
- A sorologia para mensuração de anticorpos IgG contra o sonicado de glândula salivar do vetor *Anopheles darlingi* é uma valiosa técnica que pode ser utilizada para estimar exposição ao *P. vivax* e pode também estimar a imunidade clínica;
- A exposição natural ao vírus da hepatite B está associada à redução da gravidade clínica da malária causada tanto pelo *P. vivax* quanto pelo *P. falciparum*;
- A malária grave causada pelo *P. vivax* apresenta uma intensa e desregulada resposta inflamatória sistêmica;
- A enzima superóxido dismutase-1 é um marcador potencial de gravidade na infecção pelo *P. vivax* e provavelmente está envolvida na patogênese da malária

grave onde há liberação de quantidades elevadas de heme livre. Os níveis desta enzima podem ser estimados por um método simples facilmente aplicável ao SUS.

O conjunto de manuscritos que compõem a tese acrescenta importantes informações sobre os determinantes da malária. Tais conhecimentos poderão servir para fundamentar investimentos futuros na área de controle da malária no Brasil.

8. REFERÊNCIAS

Aqui estão listadas as referências utilizadas na introdução e discussão geral da tese. As referências citadas apenas nos manuscritos não estão listadas nesta seção.

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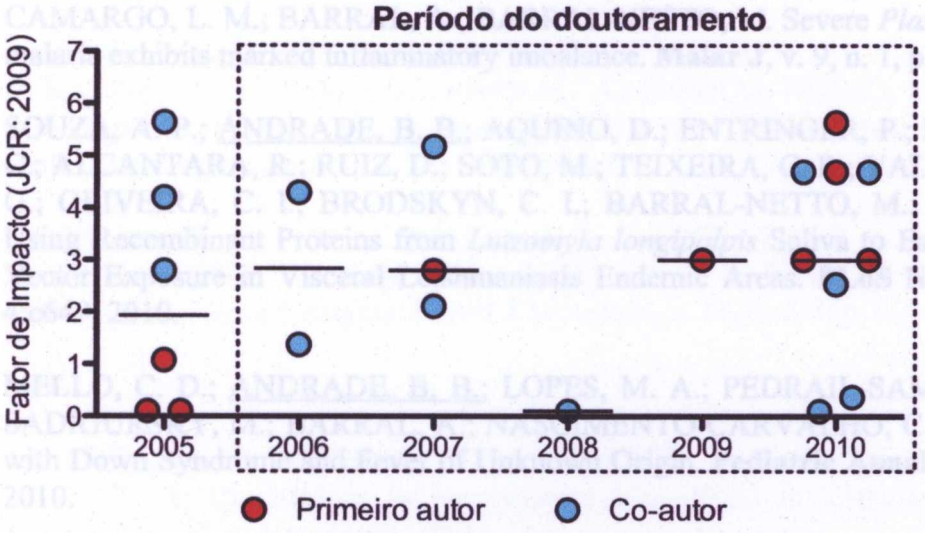
9. ANEXO I: PRODUÇÃO CIENTÍFICA NO DOUTORADO

Desempenho do estudante quanto à produção científica:

O estudante iniciou os trabalhos no Laboratório Integrado de Microbiologia e Imunoregulação em março de 2002, como bolsista de iniciação científica do CNPq. Até 28 de Março de 2010, o estudante foi autor de 22 trabalhos publicados em periódicos, incluindo 9 como primeiro autor e 13 colaborações. A figura abaixo ilustra a produção em todo o período. Em seguida há uma listagem de todos os trabalhos já aceitos que foram produzidos no período do doutorado. Há ainda 01 manuscrito desta tese em análise em revistas e outros 5 frutos de colaborações que ainda não foram aceitos até Março de 2010.



2002-2005: Iniciação científica (bolsista CNPq) - 3 anos / total de publicações: 6
2006-2010: doutorado (bolsista CNPq) - 4 anos / total de publicações: 16

Média de publicações por ano: 3.14
Estatísticas feitas em Junho de 2010:
- Total de publicações listadas no Web of Science: 15
- Total de citações (Web of Science): 98
- Média de citação por artigo(Web of Science): 6.53
- Fator H: 6.0
- Média do fator de impacto das publicações: 2,924
- Mediana do fator de impacto das publicações: 3,002

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