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SUSCETIBILIDADE GENÉTICA E FARMACOGENÉTICA DA MALÁRIA CAUSADA POR *P. VIVAX* NA AMAZÔNIA BRASILEIRA

VINICIUS DE ALBUQUERQUE SORTICA

Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para obtenção do grau de Doutor em Ciências

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End of the road
"I won't be the last
I won't be the first
Find a way to where the sky meets the earth
It's all right and all wrong
For me it begins at the end of the road
We come and go..."

(Eddie Vedder)

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SUMÁRIO

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

ABC - "ATP-binding cassete"

ACT - terapia combinada baseada em artemisinina

ADR - efeito adverso

BCRP - Proteína de resistência relacionada ao câncer de mama ("breast cancer resistance protein")

CD - células dendríticas

CYP - Citocromo P450

DNERu - Departamento Nacional de Endemias Rurais

G6PD - glicose-6-fosfato desidrogenase

Ig - Imunoglobulina

IL - interleucina

INF-γ - interferon-γ

MDR - proteína de resistência a múltiplos fármacos

MHC I - complexo principal de histocompatibilidade de classe I

MHC II - complexo principal de histocompatibilidade de classe II

MRP - Proteína relacionada a resistência a multiplos fármacos

NAT - N-acetiltransferase

OATP - polipeptídio transportador de ânions orgânicos

OMS - Organização Mundial da Saúde

Pg-P - glicoproteína-P

PNCM - Programa Nacional de Controle da Malária

Th - T auxiliares (helper)

Th1 - T auxiliares do tipo 1

Th2 - T auxiliares do tipo 2

Th17 - T auxiliares do tipo 17

TNF-α - fator de necrose tumoral-α

UGT - UDP-glucuronosiltransferase

RESUMO

A malária é uma das doenças infecciosas mais graves que afligem a espécie humana, sendo endêmica na maioria das regiões tropicais e subtropicais do mundo. Em humanos, essa doença é causada pelos protozoários *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax* e *Plasmodium knowlesi*, que são transmitidos ao hospedeiro pela picada dos mosquitos do gênero *Anopheles*. No Brasil, 87% dos casos de malária ocorrem pela infecção por *P. vivax* e as infecções por *P. falciparum* e infecções mistas representam o restante.

As infecções por *P. vivax* foram tradicionalmente relacionadas com casos mais brandos de malária e pesquisas sobre essas infecções foram por muito tempo negligenciadas pela comunidade científica e indústrias farmacêuticas. Atualmente, casos severos e mortes por essas infecções são cada vez mais frequentes, tornando essa espécie de *Plasmodium* um importante alvo para os programas de controle e erradicação da malária. Apesar das evidências mostrarem que há variabilidade entre os indivíduos na suscetibilidade e resposta ao tratamento da malária, estudos relacionados com a influência genética da resposta imunológica na suscetibilidade ou resistência nas infecções por *P. vivax* são escassos, e pesquisas sobre a variabilidade genética da resposta ao tratamento com os fármacos utilizados no tratamento dessas infecções são inexistentes. No presente trabalho, foram investigados a influência de 33 polimorfismos nos genes *IL1B, IL2, IL4, IL4R, IL6, IL8, IL10, IL12A, IL12B, IL12RB1, SP110, TNF, TNFRSF1A, IFNG, IFNGR1, VDR, PTPN22* e *P2X7* na suscetibilidade à malária e na evolução clínica dessa doença. Também foram investigados 30 polimorfismos nos genes *CYP1A2, CYP2C8, CYP2C9, CYP3A4, CYP3A5, SLCO1A2, SLCO1B1, SLCO1B3* e *SLCO2B1* na resposta ao tratamento da malária. Entre os anos de 2002 e 2009, 216 pacientes naturais do estado do Pará diagnosticados com malária causada por *P. vivax* aceitaram participar do estudo. Todos os pacientes fizeram o tratamento padrão com cloroquina associada à primaquina. Desse grupo de pacientes, 167 foram avaliados clinicamente durante o período do tratamento e os níveis de parasitemia e gametocitemia foram estimados diariamente. Além dos genes em estudos, os pacientes também foram genotipados para a enzima G6PD. A ancestralidade genética desses pacientes foi estimada por um conjunto de 48 marcadores de ancestralidade.

O presente trabalho descreve a associação de polimorfismos nos genes *IL1B, IL4R, IL12RB1* e *TNF* com a suscetibilidade à malária, e dos polimorfismos nos genes *IL6, IL12B* e *VDR* aos níveis de parasitemia e gametocitemia. Já as variantes nos genes *IL6* e *IL10* foram associadas à severidade dos sintomas nas infecções por *P. vivax*. Esse trabalho, também demonstra que alelos dos genes *CYP2C8, ABCB1, SLCO1B1* e *SLCO2B1* influenciam a resposta ao tratamento da malária. Variantes nesses genes estão associadas a uma resposta mais rápida à medicação fazendo com que seus portadores eliminem os parasitos e gametócitos em menor tempo.

Esses resultados contribuem para a compreensão da malária e podem ajudar na obtenção de métodos de controle e esquemas terapêuticos mais eficientes para o controle dessa doença.

ABSTRACT

Malaria is a major infection disease that affects human species, and is spread in tropical and sub-tropical regions in different continents. This disease is caused by *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium knowlesi* protozoan which are transmitted to humans by *Anopheles* mosquito bites. In Brazil, 87% of malaria infections result from *P. vivax* infections, *P. falciparum* and mixed infections represent all other cases.

P. vivax infections were traditionally related to malaria milder symptoms therefore research in such infections was neglected by the scientific community and pharmaceutical industries. At present severe cases and deaths by these infections are more frequent and *P. vivax* become an important target for malaria control and elimination program. Despite evidences showing malaria susceptibility and treatment response variability, studies in immune system response related to *P. vivax* malaria susceptibility are scarce. In the present study 33 polymorphisms in *IL1B, IL2, IL4, IL4R, IL6, IL8, IL10, IL12A, IL12B, IL12RB1, SP110, TNF, TNFRSF1A, IFNG, IFNGR1, VDR, PTPN22* and *P2X7* genes were investigated for association with malaria susceptibility, and its clinical aspects. Thirty polymorphisms in *CYP1A2, CYP2C8, CYP2C9, CYP3A4, CYP3A5, SLCO1A2, SLCO1B1, SLCO1B3* and *SLCO2B1* genes were investigated in relation to malaria treatment outcomes. Two hundred and sixteen (216) patients diagnosed with *P. vivax* malaria recruited during the period 2002-2009, who were born in Pará state, Brazil were enrolled in the study. All patients received chloroquine and primaquine standard treatment regimens. From these group of patients, 167 were clinically evaluated during treatment and have parasitemia and gametocytemia levels estimated daily. Besides the investigated genes, the patients were genotyped for G6PD. Genetic ancestry was estimated by a set of 48 ancestry markers.

The present work reports that *IL1B, IL4R, IL12RB1* and *TNF* gene polymorphisms were associated with malaria susceptibility, whereas *IL6, IL12B* and *VDR* gene polymorphisms were associated with parasitemia and gametocytemia levels. *IL6* and *IL10* genetic variants were associated with malaria symptoms intensity in *P. vivax* infections. The present work also reports the influence of *CYP2C8, ABCB1, SLCO1B1* and *SLCO2B1* alleles on malaria treatment response. Genetic variants in these genes were associated with

a faster drug response and patients with these variants present parasites and gametocytes clearance in a shorter time.

These results have a great value for malaria understanding and could help to improve disease control and therapeutic regimen to more efficient methods to control this disease.

CAPÍTULO 1
INTRODUÇÃO

1.1Malária

1.1.1 Aspectos Gerais

A malária é uma das doenças infecciosas mais antigas que afligem a espécie humana. Sua origem é possivelmente anterior às linhagens de primatas que precederam os hominídeos (Schlagenhauf, 2004). Relatos descrevendo epidemias de febre recorrentes em conjunto com esplenomegalia são atribuídos a malária e encontrados em antigos textos chineses (2700 a.C.), e posteriormente em outras civilizações antigas como a egípcia, indiana, grega e romana (Cox, 2002; Carter e Mendis, 2002; Retief e Cilliers, 2005). Entretanto, somente em 500 a.C., o primeiro registro descrevendo os sintomas dessa doença foi realizado por Hipócrates (460 - 377 a.C.), que descreveu os sintomas de calafrios, suor e os padrões de febre denominados como quotidianas (diárias), terçãs (a cada dois dias) e quartãs (a cada três dias) (Cunha e Cunha, 2008). Por muitos anos a malária foi descrita por diferentes nomes (febres terçã e quartã, paludismo, febre dos pântanos, entre outras) e atribuída a gases venenosos emanados dos terrenos pantanosos que envenenavam as pessoas. Essa crença originou a denominação moderna dessa doença das palavras em italiano mal' aria ("ar ruim") (Sallares, 2004; Crotti, 2005). Somente em 1880, o cientista Alphonse Laveran descreveu os parasitas no sangue de militares com malária e, posteriormente, postulou que as distintas formas visualizadas no sangue dos enfermos representariam múltiplas formas do mesmo agente etiológico (Laveran, 1893). Nos anos seguintes, a periodicidade das febres terçã e quartã foi relacionada com o ciclo de replicação dos parasitas no sangue, e as febres, com padrões irregulares, seriam decorrentes de infecções por diferentes espécies de parasitas e/ou infecções mistas (Golgi, 1889; Machiafava e Bignami, 1894). Apesar de relatos antigos, entre 500 e 221 a.C., de que a malária seria causada pela picada de insetos e mosquitos (Cox, 2002; Sallares, 2004), somente em 1910 foi demonstrado que os parasitas da malária eram inoculados em humanos pela picada de mosquitos infectados (Ross, 1910).

A malária representou um grande impacto na história do homem, sendo um fator importante em guerras, nas migrações humanas, e no desenvolvimento e declínio de diferentes civilizações (Carter e Mendis, 2002; Neghina *et al*., 2010). Acredita-se que a malária teve sua dispersão junto com os movimentos migratórios dos homens a partir do vale do rio Nilo para a região do Mediterrâneo, depois para Ásia e Europa. A chegada da malária no continente americano é atribuída à chegada dos conquistadores espanhóis à

América Central, e posteriormente ao tráfico de escravos. A infestação por malária na América determinou que a doença atingisse uma distribuição mundial no início dos anos 1800 (Sherman, 1998; Schlagenhauf, 2004; Neghina *et al*., 2010). Atualmente, ela continua disseminada pelos continentes americano, africano, asiático e europeu (Figura 1).

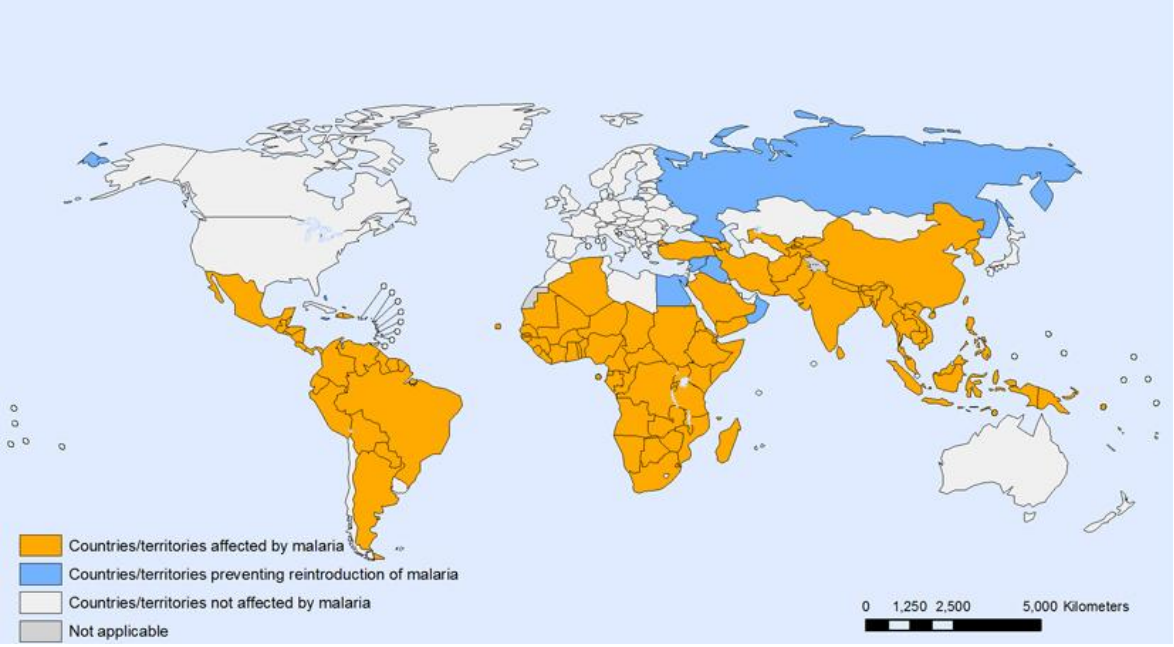

Figura 1 - Mapa mundial demonstrando os países e territórios afetados pela malária no ano de 2010 (World Health Organization, 2012a).

A malária em humanos tem como agentes etiológicos cinco espécies de protozoários do gênero *Plasmodium* (*Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax* e *Plasmodium knowlesi*) que são transmitidos ao hospedeiro pela picada dos mosquitos do gênero *Anopheles*. Das espécies de *Plasmodium* que infectam os humanos, o *P. falciparum* apresenta a maior taxa de mortalidade, possuindo uma alta incidência no continente africano, enquanto o *P. vivax* apresenta a maior distribuição geográfica entre as espécies sendo responsável por 60 a 80% dos casos de malária nos continentes asiático e americano. Em conjunto esses agentes representam mais de 95% dos casos de malária registrados (Mueller *et al*., 2009; World Health Organization, 2012b). O *P. malariae*, está presente na maioria das áreas endêmicas de malária, tendo uma prevalência de até 30% em algumas regiões da África, entretanto sua prevalência em outras regiões não ultrapassa 2% dos diagnósticos. O *P. ovale* possui uma

distribuição geográfica muito mais restrita, estando presente no continente africano e algumas regiões da Ásia e Oceania, apresentando uma prevalência de 3% a 10% dos diagnósticos nessas regiões (Mueller *et al.,* 2007a; 2007b). *P. knowlesi* é um parasita que originalmente infecta macacos do gênero *Macaca* no sudoeste da Ásia, mas atualmente apresenta uma prevalência importante de casos de malária humana nesta região (Chin *et al.,*1965; Singh *et al.,* 2004; Cox-Singh, 2012).

Apesar dos esforços internacionais para o combate e a erradicação dessa doença, a Organização Mundial da Saúde (OMS) estima que no ano de 2010 tenham ocorrido 219 milhões de casos e ao menos 660 mil mortes no mundo (World Health Organization, 2012b). No ano de 2011, aproximadamente 80% dos casos ocorridos e 90% das mortes causadas por essa doença foram observados no continente africano. Nas Américas, aproximadamente 30% da população de 21 países reside nas áreas de transmissão da malária. Entre os casos relatados no continente americano nos últimos anos, o Brasil representa aproximadamente metade das incidências (Lacerda *et al.,* 2012; World Health Organization, 2012b).

No Brasil, a malária ocorre pela infecção dos protozoários *P. falciparum*, *P. malariae* e *P. vivax,* e as primeiras descrições de casos autóctones dessa infecção datam do século XVI, com o início da colonização européia. No final do século XIX e início do século XX, a malária estava disseminada por todos os estados do Brasil (Barcelar, 1963; Camargo, 2003). Nesse período, grandes epidemias surgiram devido aos movimentos migratórios para a região amazônica durante os ciclos de exploração de borracha e minério no país.

A morbidade determinada pela malária desde sua introdução no Brasil esteve sob oscilações. Na década de 1950, com a criação do Departamento Nacional de Endemias Rurais (DNERu) e intensas campanhas para o controle da malária, essas infecções começaram a ser controladas nas regiões sul, sudeste, nordeste e centro-oeste. Entretanto, apesar das campanhas de controle e da diminuição na área de transmissão da doença, no final da década de 1960, com a resistência do *P. falciparum* à cloroquina, a morbidade aumentou consideravelmente no país. A incidência de *P. falciparum* atingiu seu ápice na década de 1980 (Marquez e Gutierrez, 1994; Loiola *et al.,* 2002; Coura *et al.,* 2006). No final da década 1980, devido a um controle mais efetivo das autoridades de saúde no Brasil, e à utilização de novos esquemas terapêuticos, o número de casos de malária por *P.*

falciparum diminuíram consideravelmente em relação ao número de casos de malária por *P. vivax* (Figura 2). Com a intensificação do Programa Nacional de Controle da Malária (PNCM) do Ministério da Saúde em conjunto com a OMS, no final da década de 1990, a incidência dessa doença no país tem diminuído nos últimos anos (Ministério da saúde, 2008; Oliveira-Ferreira *et al.,* 2010; World Health Organization, 2012b).

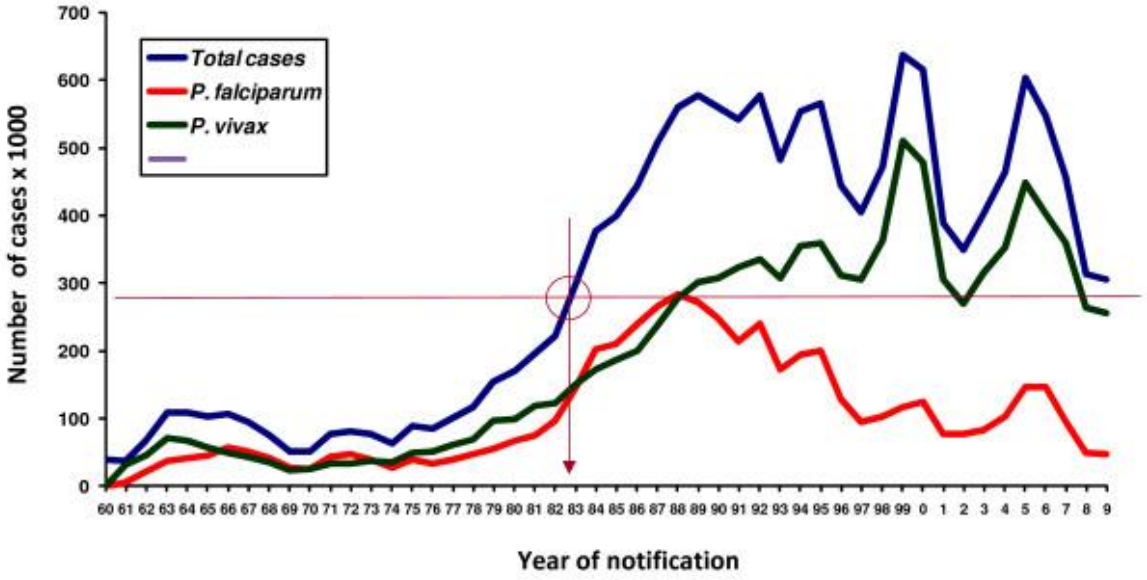

Figura 2 - Casos de malária registrados na Amazônia brasileira entre 1960 e 2009 de acordo com as espécies de *Plasmodium* (Oliveira-Ferreira *et al.,* 2010).

Atualmente, as infecções por malária concentram-se nos municípios da região da Amazônia Legal, que compreende os estados do Acre, Amazonas, Amapá, Maranhão, Mato Grosso, Pará, Rondônia, Roraima e Tocantins, onde são uma importante causa de morbidade na população (Figura 3) (Silva-Nunes *et al.,* 2008; Ferreira and Silva-Nunes, 2010). Os últimos dados do Ministério da Saúde mostram que as infecções por *P. vivax* representam 87% dos casos de malária no país e que as infecções por *P. falciparum* e infecções mistas representam o restante. No ano de 2011, o país notificou 267.045 casos confirmados de malária (231.618 causados pelo *P. vivax*) na região amazônica e 70 mortes (World Health Organization, 2012b).

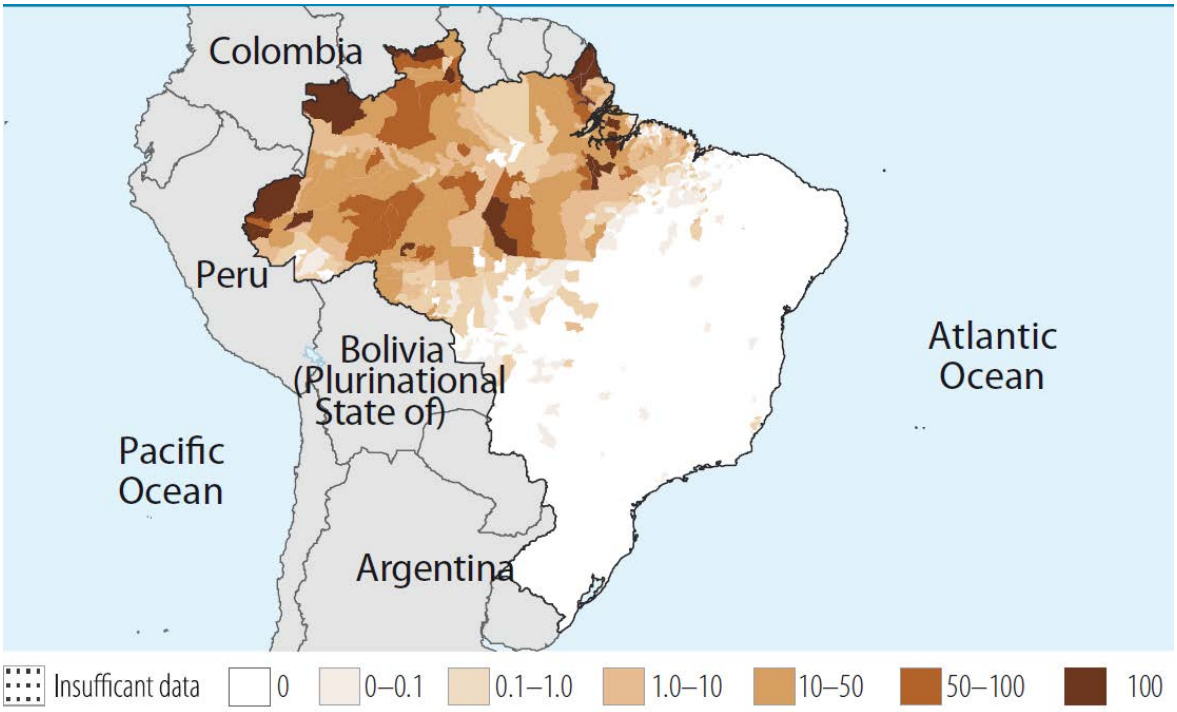

Figura 3 - Distribuição de casos/1000 indivíduos confirmados de malária no Brasil no ano de 2011 (World Health Organization, 2012b)

1.1.2 Ciclo Biológico (*Plasmodium spp*.)

A infecção por malária em humanos começa com a picada das fêmeas de mosquito do gênero *Anopheles* que inocula centenas de esporozoítos na corrente sangüínea do hospedeiro. Os esporozoítos eventualmente migram para o fígado formando vacúolos parasitóforos nos hepatócitos do hospedeiro. Neste estágio, eles podem permanecer latentes como hipnozoítos (o que geralmente ocorre nas infecções por *P. vivax* e *P. ovale)*, ou se desenvolver em milhares de merozoítos em vacúolos chamados merossomos. Após o descolamento dos merossomos para a corrente sangüínea, os merozoítos, rapidamente, invadem os eritrócitos onde replicam, iniciando a fase eritrocitária que corresponde ao ciclo de febre e calafrios da malária. Alguns parasitas se diferenciam em gametócitos masculinos e femininos que podem viver quiescentes na corrente sangüínea durante semanas, sendo muitas vezes sugados por mosquitos que se alimentam do sangue desse hospedeiro. Ao serem sugados pelos mosquitos, os gametócitos masculinos e femininos sofrem uma rápida transição em gametas ativos e se unem, formando o oocineto (forma parasita diplóide móvel e de curta duração). O oocineto migra até a parede do intestino médio do mosquito onde forma o oocisto. Após divisões meióticas, novos esporozoítos são

formados no oocisto. Quando ocorre a ruptura do oocisto, os esporozoítos migram para a glândula salivar do mosquito onde aguardam a transferência para o hospedeiro vertebrado (Figura 4) (Aly *et al.*, 2009; Lindner *et al.*, 2012).

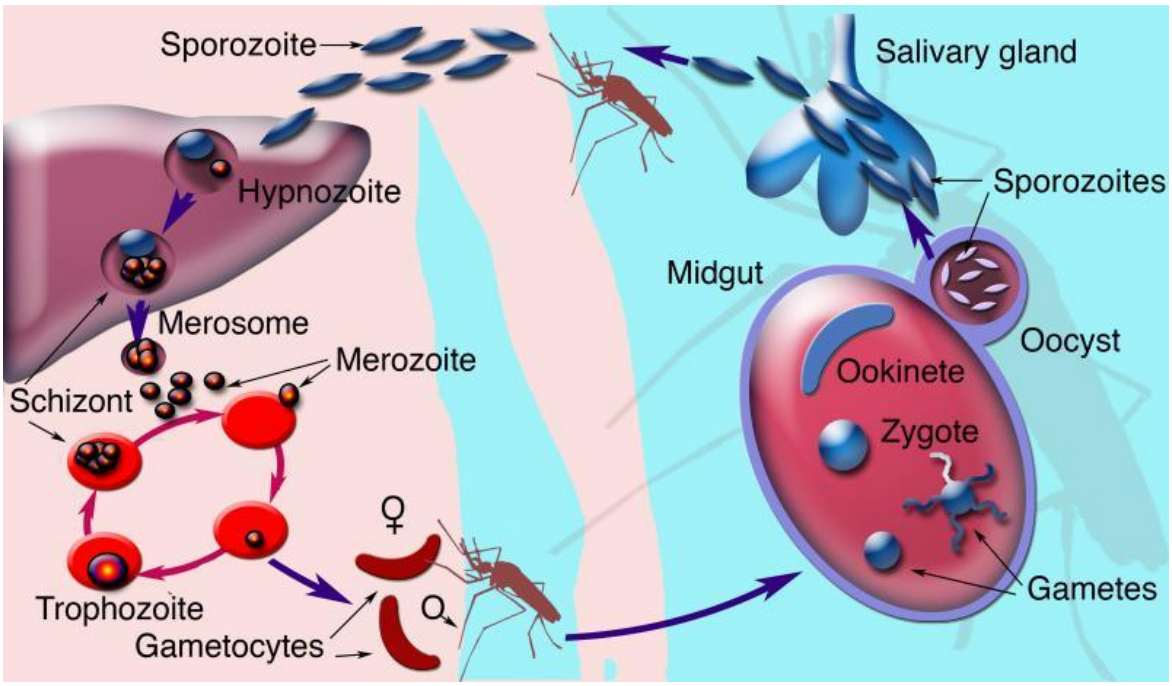

Figura 4 - Diagrama do ciclo biológico do *Plasmodium* (Winzeler, 2008).

1.1.3 Apresentação Clínica

Após ser infectado com esporozoítos, o paciente pode ficar assintomático por aproximadamente uma semana ou apresentar sintomas leves, irregulares e não característicos. Esse período coincide com o estágio de multiplicação pre-eritrocitário do parasito no fígado. Posteriormente, quando os merozoítos começam a fase eritrocitária, os indivíduos infectados começam a apresentar os sintomas mais característicos da malária como a anemia, esplenomegalia, febre alta, calafrios e sudorese (paroxismo). Durante o curso da doença os pacientes podem apresentar outros sintomas como dores pelo corpo (mialgias, artralgias, lombalgias) anorexia, icterícia, náusea, vômitos, diarreia e cefaleia. A apresentação dos sintomas e a gravidade da doença podem variar dependendo da espécie de plasmódio, da imunidade prévia do hospedeiro aos parasitas, da idade e do estado de saúde do paciente (Garcia, 2010; Bartoloni e Zammarchi, 2012; Anstey *et al.*,2012). As principais características da malária causada pelas diferentes espécies de plasmódio estão resumidas na Tabela 1.

Os *P. vivax* e *P. ovale* apresentam infecções com sintomas clínicos semelhantes. Após o período de incubação, os pacientes começam a apresentar os sintomas com uma periodicidade de 48h (a cada 3 dias; febre terçã). A infecção por esses parasitas geralmente não ultrapassa 2% dos eritrócitos, uma vez que eles infectam preferencialmente eritrócitos jovens, limitando a infecção e causando uma anemia mais branda. Os *P. vivax* e *P. ovale* possuem a característica de ficar latentes no fígado dos hospedeiros na forma de hipnozoítos, e, posteriormente à infecção primária, podem ocasionar recaídas de malária nesses indivíduos. As recaídas podem acontecer semanas, meses e até anos após a infecção primária, e os sintomas são geralmente iguais aos da primeira infecção (Collins e Jeffery, 2005; Anstey *et al.*,2009; Anstey *et al.,* 2012). Infecções por *P. vivax,* atualmente, apresentam casos severos e fatais tão frequentes quanto os causados por *P. falciparum* em algumas regiões, com complicações como a ruptura do baço, convulsões, icterícia, problemas renais, anemia severa, coma e síndrome de dificuldade respiratória (Kochar *et al.,* 2005; Saleri *et al.,* 2006; Anstey *et al.,* 2007; Kochar *et al.,* 2007; Genton *et al.,* 2008; Tjitra *et al.,* 2008; Kochar *et al.,* 2009). O *P. ovale* raramente causa infecções severas de malária; entretanto, casos apresentando a síndrome de dificuldade respiratória já foram relatados (Lee and Maguire, 1999; Rojo-Marcos *et al.,* 2008).

O *P. falciparum* é o responsável pela maioria dos casos graves e mortes por malaria, principalmente em crianças (World Health Organization, 2012b). As infecções por esses parasitas apresentam febre inicial irregular e sintomas parecidos com uma gripe e, posteriormente, febre e paroxismo periódicos diários (febre quotidiana), a cada três dias (febre terçã), ou em intervalos de 36 horas (febre semi-terçã). O *P. falciparum* infecta eritrócitos em todos os estágios de desenvolvimento e a proporção de células infectadas pode ultrapassar 50% dos eritrócitos. As células infectadas por *P. falciparum* mudam sua forma e adquirem a capacidade de capturar eritrócitos não infectados criando aglomerados que causam a obstrução vascular e a adesão aos tecidos (citoaderência) causando danos aos órgãos (Silamut *et al.,* 1999; Pouvelle *et al.,* 2000) e complicações graves

Os sintomas graves comuns a essas infecções são principalmente febre alta, anemia severa, malaria cerebral, insuficiência renal, insuficiência respiratória e disfunção hepática e quando não tratados adequadamente são, muitas vezes, fatais (Kumar *et al.,* 2007; Bartoloni e Zammarchi, 2012).

Tabela 1 - Características clínicas das cincos espécies de *Plasmodium* que infectam humanos (Modificado de Garcia, 2010)

Infecção	*P.vivax*	*P. ovale*	*P. malariae*	*P. falciparum*	*P. knowlesi*
Período de Incubação	8-17 dias	10-17 dias	18-40 dias	8-11 dias	9-12 dias
Sintomas Prodrômicos					
Severidade	leve a moderada	moderada	leve a moderada	leve	leve a moderada
Padrão de Febre inicial	irregular (48h)	irregular (48h)	regular (72h)	remitente contínuo	regular (24h)
Periodicidade de sintomas	48h	48h	72h	36-48h	24-27h
Paroxismo inicial					
Severidade	moderada a severa	leve	moderada a severa	severa	moderada a severa
Duração média	10h	10h	11h	16-36h	indeterminado
Duração primeiro ataque*	3-8 ou + semanas	2-3 semanas	3-24 semanas	2-3 semanas	indeterminado
Duração infecção*	5-7 anos	12 meses	20 ou mais anos	6-17 meses	indeterminado
Infecção de eritrócitos	jovens	jovens	velhos	todos os tipos	todos os tipos
Anemia	leve a moderada	leve	leve a moderada	severa	moderada a severa
Infecção no SNC[#]	rara	possível	rara	comum	possível
Síndrome nefrótica	possível	rara	comum	rara	provavelmente comum

*sem tratamento

Sistema Nervoso Central

Infecções por *P. malariae* apresentam sintomas geralmente brandos e raramente apresentam complicações. Os sintomas iniciais são parecidos com as infecções por *P. vivax*, porém o padrão de febres e paroxismo ocorre em um intervalo de 72 horas (febre quartã). Esses parasitos infectam preferencialmente eritrócitos velhos e por isso a parasitemia raramente excede 1% dos eritrócitos, autolimitando a infecção. Essas infecções são frequentemente associadas à síndrome nefrótica, que é a complicação mais comum dessa infecção (Collins and Jeffery, 2007).

O *P. knowlesi* foi recentemente identificado como agente etiológico da malária humana, e apresenta sintomas parecidos com as infecções por *P. vivax* e *P. falciparum*, podendo ocorrer hiperparasitemia. Apesar de apresentar uma distribuição geográfica distinta, essas infecções podem ocasionar sintomas brandos a severos, sendo as complicações mais comuns as insuficiências respiratória, renal e hepática (Daneshvar *et al.,* 2009, Cox-Singh *et al.,* 2010; Cox-Singh, 2012).

1.1.4 Resposta Imunológica

Os processos envolvidos na resposta imunológica da malária não são completamente compreendidos. Entretanto, diversos estudos demonstram que um balanço sensível entre as respostas pró e anti-inflamatórias, mediada por citocinas produzidas por células T auxiliares ("helper" - Th) e macrófagos, é necessário para uma resposta imunológica adequada à malária.

Em modelos animais, foi demonstrado que as células dendríticas (CDs) são responsáveis pela apresentação de antígenos, indução e produção citocinas, promovendo a resposta contra o parasita. Na infecção inicial, quando a densidade de parasitas é baixa, as CDs apresentam peptídeos derivados do antígeno através do complexo principal de histocompatibilidade (MHC de classe I e MHC de classe II) aos linfócitos T virgens, e liberam a interleucina (IL) 12 e o fator de necrose tumoral-α (TNF-α) induzindo a formação de linfócitos T CD4+ produtores de interferon-γ (INF-γ). Esse fenótipo de CDs pode ser mantido durante todo o curso da infecção por cepas murinas não letais de plasmódio (Wykes *et al.,* 2007a). No curso mais avançado da infecção ou em infecções por cepas murinas letais, a densidade de parasitas é muito alta e as CDs ficam refratárias aos estímulos para realizar a apresentação do antígeno aos linfócitos T. Desse modo, as CDs não liberam a IL-12 e o TNF-α e começam a produzir IL-10 e IL-4 (Wykes *et al.,* 2007b).

A liberação da IL-10 e IL-4 coincide com a mudança de um perfil de resposta Th1 pró-inflamatório para um perfil Th2 anti-inflamatório, diminuindo a resposta contra os antígenos da malária, bem como a outras infecções (Artavanis-Tsakonas *et al.,* 2003).

Em infecções humanas, a maioria dos estudos investigaram infecções por *P. falciparum* em regiões endêmicas e indicam que uma resposta rápida pró-inflamatória mediada por um conjunto de células Th1 e macrófagos ajuda a reduzir a parasitemia. Entretanto, uma resposta anti-inflamatória secundária mediada por um conjunto de células Th2 e monócitos é necessária para controlar a malária evitando sintomas mais graves e danos aos órgãos do paciente. O tempo e a intensidade entre as respostas pró e anti-inflamatórias mediadas por citocinas e a auto regulação desse sistema podem influenciar tanto a patologia da infecção quanto a sua progressão (Good *et al.,* 2005; Riley *et al.,* 2006; Langhorne *et al.,* 2008).

1.1.5 Tratamento

Segundo a OMS, o tratamento adequado da malária deve ser prescrito com base na gravidade da doença, a espécie de plasmódio, a idade do paciente, o histórico de infecções anteriores e a susceptibilidade dos parasitas aos antimaláricos convencionais, evitando atraso no efeito terapêutico e o agravamento do quadro clínico (Ministério da Saúde, 2010; World Health Organization, 2010).

Atualmente, diversos fármacos antimaláricos (Cloroquina, Amodiaquina, Primaquina, Quinina, Mefloquina, Halofantrina, Artemisinina, Artesunato, Artemeter, Doxiciclina) são utilizados em diferentes países, dependendo da ocorrência de cepas resistentes a esses fármacos (World Health Organization, 2010). Os fármacos antimaláricos podem atuar em diferentes fases da infecção: fármacos hipnozonticidas atuam nos hipnozoítos latentes no fígado, os fármacos esquizonticidas atuam na fase eritrocitária da infecção e os fármacos gametocíticos agem bloqueando a transmissão da doença para os vetores.

A OMS recomenda a utilização da terapia combinada baseada em artemisinina (ACTs) como a primeira linha de tratamento contra infecções de *P. falciparum* não complicada. As ACTs são terapias que possuem ao menos um fármaco derivado de artemisinina, sendo cinco esquemas recomendados: (1) artemeter em conjunto com lumefantrina, (2) artesunato em conjunto com amodiaquina, (3) artesunato em conjunto

com mefloquina, (4) artesunato em conjunto com sulfadoxi-pirimetamina, e (5) diidroartemisinina em conjunto com piperaquine. Nas infecções por *P. vivax* e *P. ovale*, a OMS recomenda como primeira linha de tratamento a combinação de cloroquina e primaquina, com a utilização de primaquina por 14 dias. Em regiões em que cepas desse parasita são resistentes à cloroquina, é recomendado o uso de ACT, embora não existam ainda muitos estudos avaliando o uso desses medicamentos contra essa espécie de parasita. Para casos de malária severa por *P. falciparum* e *P. vivax*, o tratamento recomendado é o de derivados de artemisinina por via intravenosa como primeira linha e quinina por via intravenosa como segunda linha de tratamento. A dose recomendada para cada esquema terapêutico varia conforme a idade e peso do paciente, e também nos casos de pacientes gestantes (World Health Organization, 2010).

No Brasil, nas infecções por *P. vivax*, o tratamento preconizado é a associação de cloroquina e primaquina em um tratamento de 7 dias com a mesma dose do tratamento recomendado em 14 dias. O Ministério da Saúde adotou essa medida para aumentar a adesão ao tratamento completo. Para as infecções por *P. falciparum*, o tratamento com artemeter associado com lumefantrina ou artesunato associado com mefloquina são utilizados como esquemas preferenciais. Nas infecções mistas, prescreve-se o tratamento para *P. falciparum* combinado com a primaquina, que é efetiva contra os gametócitos e hipnozoítos do *P. vivax* (Ministério da Saúde, 2010).

1.1.5.1 Cloroquina

A partir de 1946, a cloroquina emergiu como o medicamento de escolha para o tratamento e a profilaxia de malária causada por todos as espécies de plasmódios nos países tropicais endêmicos, uma vez que era um fármaco mais eficaz e menos tóxico que a quinina e a quinidina. De 1966 em diante, o surgimento da resistência à cloroquina foi constatado mundialmente. Apesar da ampla distribuição de cepas de *P. falciparum* resistente à cloroquina, este fármaco ainda é usado como primeira linha de tratamento para *P. ovale*, *P. malariae* e *P. vivax* em regiões onde não há a prevalência de cepas resistentes (Cooper e Magwere, 2008; Sousa *et al.,* 2008).

A cloroquina é uma 4-aminoquinolina derivada de quinina e, quando administrada por via oral, é rapidamente e quase completamente absorvida a partir do trato gastrointestinal com uma biodisponibilidade de 75-80%. A concentração máxima no

plasma é alcançada em 1 a 2 horas e permanece até 3,6 horas após a administração (Augustijns e Verbeke, 1993). A cloroquina é metabolizada no fígado pelas isoformas do citocromo P450 CYP2C8, CYP3A4, CYP3A5, e em menor extensão pelo CYP2D6. O fígado metaboliza 30 a 50% da cloroquina administrada, embora sítios extra-hepáticos também possam ter significado clínico, devido à ampla distribuição tecidual da CYP3A (Ducharme e Farinotti, 1996; Kim *et al.,* 2003; Projean *et al.,* 2003). Todos os metabólitos da cloroquina são ativos, sendo o principal a N-desetilcloroquina (Gustafsson *et al.,* 1983; Ette *et al.,* 1989). Até 70% da dose ingerida de cloroquina é excretada inalterada na urina (Pussard e Verdier, 1994).

A variabilidade interindividual nas concentrações plasmáticas de cloroquina e N-desetilcloroquina foi relatada para diferentes populações da África e da Ásia, podendo ser um fator importante para falha no tratamento ou no desenvolvimento de resistência a esse fármaco (Walker *et al.,* 1983; Hellgren *et al.,* 1989; Karim *et al.,* 1992; Hellgren *et al.,* 1993; Maitland *et al.,* 1997; Mockenhaupt *et al.,* 2000; Dua *et al.,* 2000). Os transportadores de membrana MDR ("multidrug resistence") e MRP ("multidrug resistance-related proteins") responsáveis pelo efluxo celular da cloroquina em *P. falciparum* são também considerados um importante fator no desenvolvimento de resistência a cloroquina (Min *et al.,* 2007).

A cloroquina possui atividade esquizonticida para todas as espécies de plasmódio e gametocítica para os *P. vivax* e *P. malariae*, não possuindo ação contra as formas latentes no fígado (Jeffery, 1958). Além de seu efeito antimalárico, a cloroquina tem também ação antipirética e anti-inflamatória (van den Borne *et al.,* 1997; Jang *et al.,* 2006; Wozniacka *et al.,* 2007). O mecanismo de ação desse fármaco não é totalmente compreendido, todavia, sabe-se que ele inibe de maneira específica a degradação da hemoglobina pelo plasmódio elevando essa proteína para níveis tóxicos para esses parasitas (Krughak and Ginsburg, 1991). Os efeitos adversos (ADRs) graves desse medicamento em doses terapêuticas normais são raros. Já os ADRs mais comuns são o prurido, que pode ser intolerável; cefaléia, náusea, sintomas gastrointestinais e "visão turva". O enfraquecimento irreversível da visão, resultante do acúmulo de cloroquina na retina, é uma complicação rara, porém reconhecida, do tratamento por tempo prolongado (Alkadi, 2007, Cooper e Magwere, 2008).

1.1.5.2 Primaquina

A primaquina é o fármaco de escolha para a cura radical da malária por *P. vivax* desde 1950, sendo este o único fármaco disponível para prevenir as recaídas pelo parasita (Krudsood *et al.,* 2008). O mecanismo de ação da primaquina não é totalmente conhecido, mas ela é altamente ativa contra gametócitos de todas as espécies de malária, e contra hipnozoítos do *P. vivax* e *P. ovale*, embora existam variações geográficas quanto à sensibilidade de hipnozoítos a esse fármaco (Baird, 2009).

A primaquina é uma 8-aminoquinolina derivada de quinino, e é rapidamente absorvida quando administrada por via oral, porém, ocorrem variações individuais no seu perfil farmacocinético (Flether *et al.,* 1981; Kim *et al.,* 2004; Goller *et al.,* 2007). A concentração máxima no plasma ocorre dentro de 1 a 3 horas, com uma meia-vida de cerca de cinco horas (Hill *et al.,* 2006), sendo rapidamente metabolizada no fígado pelo CYP1A2 e CYP3A4 (Bangchang *et al.,* 1992; Constantino *et al.,* 1999) e somente uma pequena porção é excretada inalterada. O principal metabólito ativo da primaquina é a carboxiprimaquina (Hill *et al.,* 2006).

O mecanismo exato de ação da primaquina na eliminação dos gametócitos e hipnozoítos não é completamente compreendido, entretanto, esse fármaco possivelmente interfere na função da ubiquinona na cadeia respiratória da mitocôndria do parasito gerando metabólitos reativos com potenciais oxidativos intracelulares (Hill *et al.,* 2006; Schelsinger *et al.,* 1988).

A primaquina destrói os eritrócitos, sobretudo em pessoas com deficiência da enzima glicose-6-fosfato desidrogenase (G6PD), causando anemia hemolítica. A gravidade da anemia hemolítica parece estar relacionada com a dose do fármaco e do grau de deficiência da G6PD, e por isso a análise da atividade da G6PD é indicada em pacientes que utilizam a primaquina. Cerca de 10-12% das pessoas que utilizam a primaquina apresentam cólicas abdominais, anorexia, náuseas, vômitos ocasionais e diarreia. Outros efeitos adversos graves associados com primaquina são praticamente inexistentes, existindo apenas dois relatos de distúrbios neuropsiquiátricos (Alkadi, 2007; Vale *et al.,* 2008).

1.2 Aspectos Imunogenéticos da Suscetibilidade à Malária

As citocinas são proteínas imunomoduladoras produzidas por diferentes células do sistema imunológico e desempenham diversas funções biológicas. Uma complexa rede de interações entre as citocinas e as células de defesa do organismo representa o cerne de uma resposta imunológica efetiva contra as infecções (Goldszmid e Trinchieri, 2012). A produção de diferentes citocinas pelos linfócitos T CD4 em diferentes contextos de resposta imunológica, levou a sua classificação como linfócitos Th1, Th2, e mais recentemente em Th17 (Mosmann *et al.,* 1986; Killar *et al.,* 1987; Aggarwal *et al.,* 2003). Os linfócitos Th1 produzem predominantemente IL-2, IL-12 e IFN-γ e são importantes na imunidade mediada por células. Os linfócitos Th2 produzem IL-4, IL-5, IL-9, IL-13 e outras citocinas que promovem uma resposta imune humoral. Já os linfócitos Th17 produzem IL-17, IL-21, IL-22 e induzem uma resposta imunológica contra bactérias extracelulares e fungos além de estarem envolvidos em diferentes doenças inflamatórias crônicas (Zhu *et al.,* 2010; Pulendran e Artis, 2012). A expressão dos diferentes perfis de células Th pode exercer uma influência importante na resposta às infecções. Uma predominância de uma resposta com um perfil Th2 e a redução de um perfil Th1 está relacionada a maior suscetibilidade às doenças virais e bacterianas (Hill, 1998). Na resposta imunológica à malária, um balanço entre o perfil Th1 e Th2 parece estar envolvido nos aspectos clínicos dessa doença e no seu desfecho (Langhorne *et al.,* 2008).

Variantes alélicas nos genes relacionados com a regulação, expressão e função das citocinas que moldam o perfil Th1 e Th2 durante a resposta imunológica tem sido objeto de estudos relacionados à suscetibilidade para diferentes doenças infecciosas. Nas infecções por malária, variantes alélicas nesses genes foram principalmente estudadas em infecções por *P. falciparum*, uma vez que essas infecções apresentam um quadro mais agudo e uma alta taxa de mortalidade. Pesquisas realizadas nos últimos anos demonstraram que polimorfismos em genes de diferentes citocinas estão associados às características dessas infecções, como quadros severos, parasitemia, e suscetibilidade (Tabela 2).

Diferentes estudos relataram polimorfismos do gene *IL1B* com a sintomatologia e a severidade da malária causada por *P. falciparum* em populações da África. O polimorfismo de nucleotídeo único (SNP) 3935C>T foi associado com os níveis de parasitemia em crianças de Gana com malária não complicada e com a malária cerebral em um estudo de caso-controle realizado em Gâmbia (Gyan *et al.,* 2002; Walley *et al.,* 2004).

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Tabela 2 - Polimorfismos de citocinas associados com susceptibilidade ou severidade de infecções por *P. falciparum* (modificado de Driss *et al.*, 2011)

Gene	SNP	Fenótipo	População	Amostra	*P*	Referência
TNF	-238A>G	↓S, ↓MC, ↓AS	Gâmbia	420	<0.001	McGuire *et al.*, 1999
	-308C>T	↓S, ↓MC, ↓AS	Gâmbia	376	0.01	McGuire *et al.*, 1994
	-238G/-308G/ -857T/-863C/-1031T	↑MC	Mianmar	245	<0.001	Ubalee *et al.*, 2001
	-1031C/-863C/-857C	↑MC	Tailândia	466	0.002	Hananantachai *et al.*, 2007
	-308C>T	↓ADF	Gâmbia	780	0.01	Atkinson *et al.*, 2008
	-1031C>T	↑S	Índia	361	0.01	Sinha *et al.*, 2008
	-863A>C	↑S	Índia	361	0.003	
	-308C>T	↑S	Vietnã	956	0.02	Dunstan *et al.*, 2012
IFNG	-183G>T	↓MC, ↓P	Mali	136	0.009	Cabantous *et al.*, 2005
	-183G>T	↓MC, ↓P	Mali	136	0.01	
IFNGR1	-56T>C	↓MC	Gâmbia	1131	0.006	Mangano *et al.*, 2008
IL1A	4845G>T	↑S, ↑MC, ↑AS	Gâmbia	376	0.03	Walley *et al.*, 2004
	340G>T	↑S	Vietnã	956	0.005	Dunstan *et al.*, 2012
IL1B	3953C>T	↑S, ↑MC, ↑AS	Gana	461	0.03	Gyan *et al.*, 2002
	3953C>T	↑P	Gana	461	0.01	
IL4	-524C>T	↑IgG total	Burkina Faso	159	0.01	Luoni et al 2001
	-589C>T	↑IgE total	Burkina Faso	580	0.01	Verra *et al*., 2004

	33T/-590T	↑IgE total e MC	Gana	476	0.03	Gyan *et al.*, 2004
	-590C>T	↑P	Tailândia	279	0.04	Tangteerawatana *et al.*, 2007
IL10	-1082G/-819C/-592C	↓AS	Kênia	375	0.04	Ouma *et al.*, 2008
	-1082G/-819C/-592C	↓IM	Moçambique	240	0.01	Zhang *et al.*, 2012a
IL10RA/IL10RB	-185T/-116T/-754A/-750C	↓S	Gabão	340	0.01	Velavan *et al.*, 2012
IL12A	rs2243140	↓AS	Quênia	913	0.006	Zhang *et al.*, 2012b
IL12B	IL12B.1/159C	Mortalidade MC	Tanzânia/Kenia	413	0.002	Morahan *et al.*, 2002
	IL12pro.1	↑MC	Mali	240 familias	<0.001	Marquet *et al.*, 2008
	IL12pro.1	↑MC	Tailândia	303	0.03	Naka *et al.*, 2009
	IL12pro.1	↑P	Tailândia	355	0.002	Phawong *et al.*, 2010
	IL12pro.2/159A	↓S	Tailândia	355	0.002	
	1188A>C	↑AS	Quênia	544	0.04	Ong'echa *et al.*, 2011
IL12RB1	rs429774	↓AS	Quênia	913	0.004	Zhang *et al.*, 2012b
	rs383483	↑P	Quênia	913	0.003	
IL22	708A>G	↓S	Gâmbia	676	0.01	Koch *et al.*, 2005
IL13	-1055C>T	↓S	Tailândia	371	0.003	Ohashi *et al.*, 2003
	431A>G	↑S	Vietnã	956	0.02	Dunstan *et al.*, 2012

S: severidade, MC: malária cerebral, AS: anemia severa, ADF: anemia por deficiência de ferro, P: parasitemia, IM: infecções

Em crianças do Kênia com malária, o haplótipo -31C/-511A de variantes no promotor do gene *IL1B* foi associado com anemia severa e com baixos níveis de IL-1β. Contudo, esses polimorfismos não foram associados com a malária cerebral em um estudo anterior realizado na Tailândia (Ohashi *et al.,* 2005; Ouma *et al.,* 2008).

Variantes alélicas na região promotora do gene IL4 foram associados com a produção de imunoglobulinas (Ig) E e G, a suscetibilidade a malaria cerebral e aos níveis de parasitemia em populações da África e da Ásia. Em Burquina Faso, os SNPs -524C>T e -589C>T foram associados com uma produção elevada de IgG e IgE. No primeiro estudo, indivíduos da etnia Fulani com malária, que são menos suscetíveis a malária nesse país, portadores do alelo -524T apresentam uma produção elevada de IgG comparado a indivíduos das etnias Mossi e Rimaibé (Luoni *et al.,* 2001). Já crianças com malária severa portadoras do alelo -589T apresentaram uma produção elevada de IgE (Verra *et al.,* 2004). Em uma população de crianças de Gana com malária cerebral, portadores do haplótipo 33T/-590T apresentaram maiores níveis de IgE, do que crianças com malária severa que não desenvolveram problemas neurológicos (Gyan *et al.,* 2004). O alelo -590T também foi associado com níveis de parasitemia em pacientes de malária severa na Tailândia (Tangteerawatana *et al.,* 2007).

Um haplótipo na região promotora -1082A/G, -819T/C e -592A/C do gene *IL10* está associado a proteção contra malaria e a anemia severa em populações africanas. Em crianças do Quênia, o haplótipo GCC foi associado com a proteção contra anemia severa e a uma maior produção de IL-10. Nesse estudo, o haplótipo ATA foi associado a um maior risco de anemia severa (Ouma *et al.,* 2008). O haplótipo GCC também foi associado em crianças de Moçambique com um menor risco de infecção por malaria no segundo ano de vida (Zhang *et al.,* 2012a).

Polimorfismos na região promotora do gene *IL12B* foram associados com a malária cerebral em estudos realizados na Tanzânia, no Mali e na Tailândia, entretanto, em um estudo realizado no Kênia esses polimorfismos não foram associados à malária cerebral (Morahan *et al.,* 2002; Marquet *et al.,* 2008; Naka *et al.,* 2009; Phawong *et al.,* 2010). A associação de diferentes polimorfismos nos genes *IL12A* e *IL12RB1* com a severidade e os níveis de parasitemia em pacientes do Kênia infectados pelo *P. falciparum* também já foram descritos (Zhang *et al.,* 2010).

Variantes genéticas na região promotora do gene *TNF* também foram associadas com sintomas e a severidade da malária causada por *P. falciparum* em diferentes populações da Àfrica e da Ásia. No Gâmbia, os SNP -308C>T e -238A>G foram associados com a severidade e com o quadro de malária cerebral, entretanto, esses resultados não foram replicados em estudos realizados no Kênia, Malaui, Índia e Tailândia (McGuirre *et al.,* 1999; Sinha *et al.,* 2008; Clark *et al.,* 2009; Kuesap *et al.,* 2010).

A malária causada por *P. vivax*, tradicionalmente relacionada com quadros mais brandos de malária, permanece, até o momento, pouco investigada quanto a essas variantes e a sua suscetibilidade genética. Trabalhos recentes demonstraram a influência de variantes dos genes do sistema imunológico nessas infecções. Na Índia, dois polimorfismos na região promotora do *TNF* (-308G>A e -1031C>T) foram associados com os níveis de TNF-α e com sintomas dos pacientes com malária, mas não com a suscetibilidade a essa doença (Sohail *et al.,* 2008). Em uma população do estado do Pará no Brasil, Medina *et al*. (2012) demonstraram a associação do SNP +874A>T no gene *IFNG* com os níveis de IFN-γ no plasma de pacientes infectados por *P. vivax*. No mesmo trabalho, o SNP -1082T>C no gene *IL10* não foi associado aos níveis de IL-10 no plasma dos pacientes com malária.

da Silva Santos *et al*. (2012) recentemente descreveram a associação de polimorfismos nos genes *IL10* e *TNF* com o risco de malária sintomática em uma região endêmica do Amazonas. Esse estudo, contudo, considerou o conjunto de infecções de diferentes agentes etiológicos em sua análise, e a influência dos SNPs -1082T>C e -376G>A nas infecções sintomáticas consideram as infecções por *P. vivax*, *P falciparum* e infecções mistas em conjunto.

Polimorfismos em genes responsáveis pela produção e expressão de citocinas e seus receptores como *IL1B*, *IL4*, *TNF*, *IFNG*, *IFNGR1*, *IL10*, *IL10RA*, *IL10RB*, *IL12B* e *IL12RB1* parecem influenciar as infecções por *P. falciparum*, porém ainda foram pouco explorados em infecções por *P. vivax*. Polimorfismos nesses genes, bem como o de outras citocinas, podem ajudar na compreensão da resposta imunológica a essas infecções. IL-6 é uma interleucina com ação tanto pró-inflamatória quanto anti-inflamatória (Scheller *et al.,* 2011) e apesar de estar associada com sintomas e com a severidade da malária, polimorfismos no gene *IL6* ainda foram pouco estudados nessa infecção (Kern *et al.,* 1989; Seoh *et al.,* 2003; Wunderlich *et al.,* 2012). Variantes alélicas em genes relacionados com proteínas envolvidas na regulação do sistema imunológico como *SP110*, *PTPN22*, *P2X7* e

VDR já foram associadas com doenças infecciosas como a tuberculose (Gomez *et al*., 2005; Tosh *et al.,* 2006; Lamsyah *et al.,* 2009; Merza *et al.,* 2009; Motsinger-Reif *et al.,* 2010; Abhimanyu *et al.,* 2011; Singla *et al.,* 2012), a dengue (Alagarasu *et al.,* 2012), a hanseníase (Goulart *et al*., 2006) e a hepatite B (Arababadi *et al*., 2010; Huang *et al.,* 2010), entretanto nunca foram investigados quanto as infecções por *Plasmodium* spp. Recentemente o polimorfismo não sinônimo 1513T>G do gene *P2X7* foi associado com a regulação da produção de citocinas durante inflamações (Wesselius *et al.,* 2012).

1.3 Aspectos Farmacogenéticos do Tratamento com Antimaláricos

A genética é um dos muitos fatores que podem afetar a resposta a medicamentos e sua contribuição pode variar de 20 a 95%, conforme o fármaco e o tipo de resposta avaliado. Os genes alvo para os estudos farmacogenéticos são os genes que codificam proteínas envolvidas na metabolização e/ou transporte dos fármacos, influenciando a farmacocinética dos compostos; os genes que codificam proteínas envolvidas no mecanismo de ação e/ou nas rotas metabólicas em que o fármaco age (farmacodinâmica); e os genes que codificam proteínas envolvidas no desenvolvimento direto da doença ou fenótipos intermediários (Meyer, 2004).

1.3.1 Genes de Metabolização

Genes envolvidos na metabolização de fármacos antimaláricos já foram descritos (Tabela 3), entretanto a frequência de variações nesses genes e a influência desses na utilização dos diferentes medicamentos ainda não foram determinadas para as regiões endêmicas de malária (Mehlotra *et al.,* 2009).

As enzimas de metabolização de fase I mais importantes pertencem a superfamília do citocromo 450 (CYP), que oxidam substâncias endógenas e xenobióticos em compostos mais hidrofílicos (Nebert e Russell, 2002). Essas enzimas são responsáveis pela metabolização da maioria dos fármacos utilizados sendo expressas principalmente no fígado (Figura 5). Os CYPs também são expressos em outros tecidos importantes como, pulmão, rins, cérebro, placenta, pele e mucosa intestinal, que é o sítio de metabolização extra-hepático mais importante (Lin e Lu, 2001; Paine *et al.,* 2006).

Tabela 3 - Principais isoformas de CYP, UGT e NAT envolvidos na metabolização de antimaláricos (modificado de Mehlotra *et al.,* 2009 e Kerb *et al.,* 2009)

Fármaco	Metabolização
Quinina	CYP3A4/3A5
Quinidina	CYP3A4/3A5
Cloroquina	CYP2C8, CYP3A4/3A5, CYP2D6
Amodiaquina	CYP2C8
Mefloquina	CYP1A2, CYP3A4/3A5
Primaquina	CYP1A2, CYP3A4/3A5
Halofantrina	CYP3A4/3A5
Lumefantrina	CYP3A4/3A5
Dapsona	CYP2C9, CYP3A4, CYP2B6, NAT2
Proguanil	CYP2C19, CYP3A4/3A5
Cloporguanil	CYP2C19, CYP3A4/3A5
Artemisinina	CYP2B6, CYP3A4, CYP2A6
Dihidroartemisina	UGT1A9, UGT2B7
Artesunato	CYP2A6, CYP2B6
Artemeter	CYP3A4/3A5
Arteether	CYP3A4/3A5, CYP2B6
Ácido Artelínico	CYP3A4/3A5

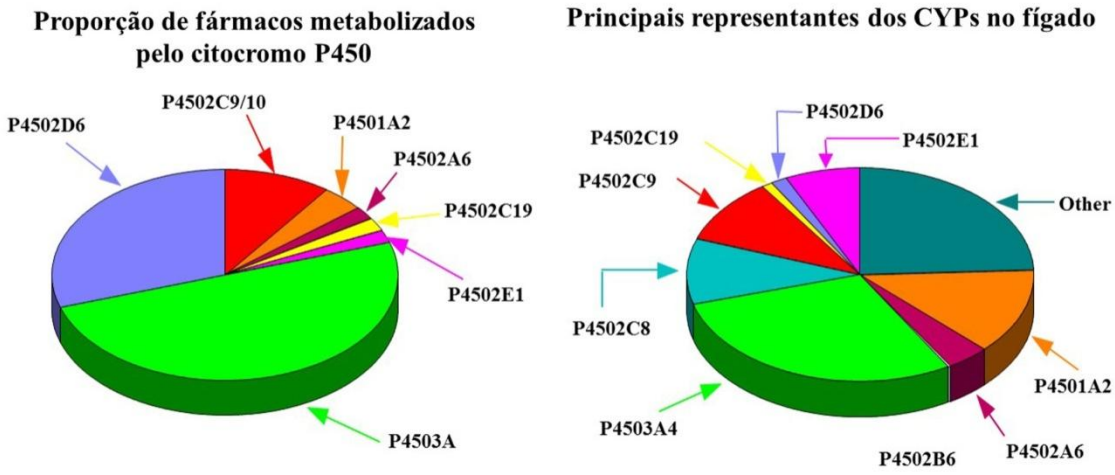

Figura 5 - Proporção de fármacos metabolizados pelo citocromo P450 e seus principais representantes no fígado (Shimada *et al.*, 1994).

Polimorfismos genéticos nas isoformas do CYP podem alterar a sua função, alterando a metabolização dos seus substratos, podendo acarretar na diferença de resposta a medicamentos, como também no risco aumentado para reações adversas (Ingelman-Sundberg *et al.*, 2007; Kirchheiner and Seeringer, 2007).

Das isoformas do CYP relacionadas com a metabolização de fármacos antimaláricos até o momento, o CYP2C8 foi o mais investigado quanto a eficácia do tratamento da malária. Os alelos *CYP2C8*2* (I269F), *CYP2C8*3* (R139K, K399R), *CYP2C8*4* (I264M) e *CYP2C8*8* (R186G) determinam redução da atividade enzimática *in vitro* (Dai *et al.*, 2001; Bahadur *et al.*, 2002; Hichiya *et al.*, 2005).

Cavaco *et al*. (2005) demonstraram que 3,6% dos pacientes com malária em Zanzibar são homozigotos para alelos de metabolização lenta, sugerindo que uma fração significativa da população que recebe tratamento possui risco mais elevado de complicações para doença e efeitos adversos com o uso da amodiaquina. Em outro estudo, Parikh *et al*. (2007) demonstraram, em Burquina Faso, que portadores dos alelos *CYP2C8*2* e *CYP2C8*3* possuem uma menor resposta ao tratamento com amodiaquina e maiores riscos de desenvolver complicações da malária.

Em um trabalho recente, Cavaco *et al*. (2012) descreveram a frequência de alelos dos genes de metabolização da amodiaquina *CYP1A1* e *CYP1B1* na população de Zanzibar. Nesse trabalho, os pesquisadores relataram uma baixa frequência de alelos para

metabolizadores rápidos, sugerindo uma baixa possibilidade da ocorrência de efeitos adversos à amodiaquina em decorrência dessas enzimas.

A influência de polimorfismos nos genes de metabolização *CYP2A6*, *CYP2B6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A4*, *CYP3A5* e *NAT2* na farmacocinética de ACTs foi descrita recentemente (Staehli-Hodel *et al.,* 2013). No trabalho, os pesquisadores avaliaram 150 pacientes da Tanzânia tratados com Artemeter associado à lumefantrina, 64 pacientes de Camboja tratados com artesunato associado à mefloquina, e 61 pacientes de Camboja tratados com diidroartemisinina associado à piperaquina. Apesar de existirem diferenças nas frequências alélicas desses genes entre as populações, nenhum dos genes foi associado a uma melhora no modelo farmacocinético. A partir dessas análises, os autores concluíram que a inclusão de dados de variantes genéticas não são necessários para uma melhora nos modelos de tratamento com ACTs, entretanto consideraram que novos estudos são necessários para replicar esses resultados em diferentes populações.

Apesar de cloroquina e primaquina comporem a primeira linha de tratamento para infecções de *P. vivax, P. ovale* e *P. malariae*, estudos farmacogenéticos avaliando a variabilidade nos genes *CYP1A2*, *CYP2C8*, *CYP2C9*, *CYP3A4*, *CYP3A5* e *CYP2D6*, responsáveis pela metabolização desses fármacos nunca foram realizados.

1.3.2 Genes de Transportadores Celulares

Transportadores de membrana são proteínas transmembranas que realizam a transposição de compostos endógenos e xenobióticos para dentro (captação) e para fora (efluxo) das células podendo exercer um papel importante na farmacocinética de medicamentos. Transportadores da família ABC ("ATP-binding cassete") e SLC ("solute carrier transporters") são os principais transportadores de efluxo e captação estudados na eficácia e no aparecimento de efeitos adversos do tratamento de diferentes fármacos (Revisões em Zair *et al.,* 2008; Sissung et al; 2010; Franke *et al.,* 2010). Até o momento, pouco se sabe sobre a influência de polimorfismos nos genes que codificam esses transportadores na farmacocinética e no tratamento de fármacos antimaláricos.

Dos transportadores de efluxo da família ABC, A glicoproteína-P (Pg-P), codificada pelo gene inicialmente denominado *MDR1* e atualmente denominado *ABCB1*, foi primeiramente descrita em células neoplásicas como a proteína responsável pela resistência ao tratamento com quimioterápicos. Dentre os polimorfismos descritos no gene

ABCB1, o 3435C>T parece ser o mais importantemente associado a efeitos farmacológicos, estando associado com a expressão dessa proteína no intestino e influenciando o transporte e a biodisponibilidade de seus substratos. Esse polimorfismo promove uma troca sinônima no éxon 26 e está em desequilíbrio de ligação com os SNPs não sinônimos: 1236C>T e 2677G>A/T (Maeda and Sugiyama, 2008). Aarnoudse *et al*. (2006) demonstraram que o haplótipo 1236T, 2677T e 3435T desse gene está associado com a predisposição a efeitos neuropsiquiátricos em mulheres que utilizam o antimalárico mefloquina. O MRP2 é um transportador codificado pelo gene *ABCC2* e expresso, principalmente, no fígado, intestino, rins, cérebro e placenta. Acredita-se que variantes desse transportador possam ter um papel importante na barreira hematoencefálica, podendo influenciar o tratamento da malária cerebral, mas até o momento nenhum estudo foi realizado. O transportador MRP4, codificado pelo gene *ABCC4*, possui uma ampla distribuição pelos tecidos, sendo expresso nos eritrócitos. A interação desse transportador com fármacos antimaláricos já foi descrita, entretanto a influencia desse transportador na resposta ao tratamento ainda não foi analisada (revisão em Kerb, 2006; Köck *et al.,* 2007). O BCRP ("breast cancer resistance protein"), codificado pelo gene *ABCG2*, é expresso nas membranas apicais de células do fígado, rins, intestino, e cérebro, e está envolvido na remoção de substâncias tóxicas dessas células, reduzindo o acúmulo de substancias nesses tecidos e a absorção. Variantes alélicas no gene *ABCG* ainda não foram investigadas no tratamento com fármacos antimaláricos (Cusatis and Sparreboom, 2008).

Os polipeptídios transportadores de ânions orgânicos (OATPs) são transportadores de captação sódio independentes codificados pelos genes da família de transportadores de soluto *SLCO* (anteriormente denominada *SLC21*) (Hagenbuch and Meier, 2004). Essas proteínas são capazes de transportar uma grande gama de ânions orgânicos anfipáticos e cátions orgânicos, como a N-metilquinidina, além de diversos xenobióticos (König *et al.,* 2006). Os OATPs são expressos em diversos tecidos e se encontram presentes em órgãos importantes como fígado, rins, intestino, cérebro e placenta (Tamai *et al.,* 2000). A influência de variantes genéticas nos genes que codificam os OATPs ainda não foram avaliadas em relação aos fármacos antimaláricos.

CAPÍTULO 2
JUSTIFICATIVA E OBJETIVOS

Doenças infecciosas tropicais, normalmente confinadas a regiões subdesenvolvidas do mundo, têm sido tradicionalmente negligenciadas pelas indústrias farmacêuticas por não serem consideradas, mundialmente, como uma prioridade em saúde, e, portanto, não conseguem grandes investimentos para sua pesquisa e para desenvolvimento de novos tratamentos. A malária causada por *P. vivax* tem sido por muito tempo uma dessas doenças. O *P. falciparum* por ser o responsável pela maioria dos casos fatais de malária com uma maior prevalência no continente africano, concentra a grande maioria dos esforços científicos e financeiros. Apesar do *P. vivax* causar pelo menos metade dos casos de malária encontrados nos demais continentes, com mais de 390 milhões de infecções por ano, pouca atenção tem sido dada para essas infecções (Baird, 2007; Price *et al*., 2007, Mueller *et al.,* 2009) em termos de pesquisa e inovação de tratamentos. O *P. vivax* foi tradicionalmente relacionado com casos mais brandos de malária, entretanto evidências dos últimos anos demonstraram que essas infecções são cada vez mais relacionadas com casos de malária severa e mortes (Barcus *et al.,* 2007; Genton *et al.,* 2008; Tjitra *et al.,* 2008; Anstey *et al.,* 2012).

A primeira linha de tratamento para malária causada por *P. vivax* é a utilização de cloroquina e primaquina desde 1946 e 1950, respectivamente. Apesar de falhas nesse tratamento e a resistência desse parasito terem sido recentemente observadas, os mecanismos de atividade e de resistência a esses fármacos ainda não são totalmente compreendidos (Baird, 2009; Baird *et al.,* 2012).

Apesar de vários estudos demonstrarem mecanismos genéticos pelos quais os *P. falciparum* e *P. vivax* criam resistência aos fármacos antimaláricos (Hayton and Su, 2008), a contribuição, da variabilidade genética em genes associados à metabolização e à farmacocinética dos antimaláricos, não foi devidamente explorada (Mehlotra *et al.,* 2009).

Estudos relacionados à suscetibilidade ou resistência à infecção pelos Plasmódios, tiveram como o principal alvo os receptores de membrana dos eritrócitos com variantes já relacionadas à suscetibilidade à malária (Beiguelman *et al.,* 2003; Verra *et al.,* 2009). Atualmente, a influência de variantes em genes relacionados à resposta imunológica contra *P. falciparum* tem sido descritos (Driss *et al*., 2011), entretanto esses genes foram pouco investigados em infecções por *P. vivax*.

Devido às características distintas entre as infecções por *P. falciparum* e as infecções por *P. vivax*, estudos específicos em infecções por esse parasita são necessários.

No Brasil, a maioria dos casos de malária é causada pelas infecções de *P. vivax*, mas as vias de ação do tratamento padrão (cloroquina e primaquina) sobre o parasito, a incidência de recaídas e ou resistência ao tratamento na região da Amazônia brasileira ainda são pouco compreendidas. Estudos relacionados com a influência da resposta imunológica na suscetibilidade ou resistência nas infecções por *P. vivax* e na variabilidade de resposta ao tratamento com os fármacos utilizados no tratamento dessas infecções são de grande importância na compreensão e atualização do tratamento da malária no Brasil.

Os objetivos do presente trabalho são:

1 – Estudar a influência na suscetibilidade à malária causada por *P. vivax* de polimorfismos em genes envolvidos na resposta imunológica.

2 – Investigar o envolvimento de variações em genes relacionados com a metabolização e farmacocinética de fármacos antimaláricos com a resposta ao tratamento da malária causada por *P. vivax*.

CAPÍTULO 3

IL1B, IL4R, IL12RB1 AND TNF GENE POLYMORPHISMS ARE ASSOCIATED WITH *PLASMODIUM VIVAX* MALARIA IN BRAZIL

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IL1B, *IL4R*, *IL12RB1* and *TNF* gene polymorphisms are associated with *Plasmodium vivax* malaria in Brazil

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Abstract

Background: Malaria is among the most prevalent parasitic diseases worldwide. In Brazil, malaria is concentrated in the northern region, where *Plasmodium vivax* accounts for 85% disease incidence. The role of genetic factors in host immune system conferring resistance/susceptibility against *P. vivax* infections is still poorly understood.

Methods: The present study investigates the influence of polymorphisms in 18 genes related to the immune system in patients with malaria caused by *P. vivax*. A total of 263 healthy individuals (control group) and 216 individuals infected by *P. vivax* (malaria group) were genotyped for 33 single nucleotide polymorphisms (SNPs) in *IL1B*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL8*, *IL10*, *IL12A*, *IL12B*, *IL12RB1*, *SP110*, *TNF*, *TNFRSF1A*, *IFNG*, *IFNGR1*, *VDR*, *PTPN22* and *P2X7* genes. All subjects were genotyped with 48 ancestry informative insertion-deletion polymorphisms to determine the proportion of African, European and Amerindian ancestry. Only 13 SNPs in 10 genes with differences lower than 20% between cases and controls in a Poisson Regression model with age as covariate were further investigated with a structured population association test.

Results: The *IL1B* gene -5839C > T and *IL4R* 1902A > G polymorphisms and *IL12RB1* -1094A/-641C and *TNF* -1031 T/-863A/-857 T/-308 G/-238 G haplotypes were associated with malaria susceptibility after population structure correction ($p = 0.04$, $p = 0.02$, $p = 0.01$ and $p = 0.01$, respectively).

Conclusion: *Plasmodium vivax* malaria pathophysiology is still poorly understood. The present findings reinforce and increase our understanding about the role of the immune system in malaria susceptibility.

Keywords: Malaria, *Plasmodium vivax*, Immune system polymorphisms, Brazilian amazon, *IL1B*, *IL4R*, *IL12RB1*, *TNF*

Background

Malaria remains one of the most important parasitic infections in the world with almost 250 million new cases diagnosed annually [1]. It is caused by infection with one or more of five species of *Plasmodium* parasites. *Plasmodium vivax* is the second most common cause of malaria in the world after *Plasmodium falciparum*, with high incidence in Asia, Central and South America causing high morbidity to these populations [2-5]. Traditionally, Brazil has been responsible for almost half of all cases of malaria in Latin America. In 2010, 334,618 cases of malaria (283,384 caused by *P. vivax*) were reported in this country [1,6].

Plasmodium vivax has unique biological features that distinguish it as a species. The most obvious features that distinguish *P. vivax* from *P. falciparum* include the development of dormant forms (hypnozoites) in the liver that cause subsequent infections in the blood called relapses, which add a substantial number of cases to the general burden of the disease and present one of the most challenging bottlenecks for vivax malaria eradication [7].

A sensitive balance between pro- and anti-inflammatory immune response, primarily mediated by cytokines released by T helper (Th) cells and macrophages, is necessary for an adequate response to malaria. An early pro-

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inflammatory response mediated by a set of Th1 cells and macrophages helps to reduce parasitaemia; however, a second anti-inflammatory response mediated by a set of Th2 cells and monocytes is required to control malaria by preventing organ damage and more severe symptoms. Timing and intensity between Th1 and Th2 response and auto-regulation can influence both pathology of infection and its progress [8-10]. Influence of the immune system gene polymorphisms with resistance/susceptibility and severity in *P. falciparum* malaria in Africa and Asia have been reported [11], however the influence of these polymorphisms in *P. vivax* infection is still poorly investigated. In patients with *P. vivax* malaria from India, two single nucleotide polymorphisms (SNP) in the *TNF* promoter (–308 G > A and -1031C > T) were associated with tumour necrosis factor (TNF) levels and clinical symptoms but not with susceptibility [12]. A recent work, demonstrated an association of +874A > T in *IFNG* gene with Interferon gamma (IFN-γ) plasma levels but not with -1082 T > C in *IL10* gene with interleukin (IL) 10 plasma levels in patients infected by *P. vivax* from Brazil [13].

Brazil has a peculiar epidemiological situation, as one of the few countries around the world with *P. vivax* predominance [6]. Data on susceptibility to *P. vivax* infection allied to several particularities of the Brazilian Amazonian region, including the diverse genetic background of its population, indicate that the generalization of findings from Southeast Asia may not be appropriate for this region [6]. The present work aims to investigate the influence of 33 SNPs in genes related to the immune system and susceptibility to *P. vivax* malaria in an Amazonian population.

Methods

Study population

A total of 216 patients were diagnosed with *P. vivax* malaria at the Evandro Chagas Institute, Belém, Pará between 2002–2009. All patients were diagnosed by thick blood smear as recommended by the Brazilian Ministry of Health [14]. The patients received the standard treatment 1,500 mg of chloroquine associated with 210 mg of primaquine in seven days. Between 2006 and 2008, 263 healthy controls were recruited at the Federal University of Pará. Unrelated subjects were interviewed at the Human and Medical Genetics Laboratory and agreed to donate DNA samples to compose a population sample for genetic studies. The individuals enrolled in the study were born in Pará state in the Brazilian Amazonian region. Patients and controls provided their written informed consent to participate in this study. The Ethics Committees of the Evandro Chagas Institute and Federal University of Pará approved the study protocol.

Genotyping

Genomic DNA was extracted from peripheral blood leukocyte subjects using proteinase K digestion and standard phenol-chloroform procedures [15]. All subjects were genotyped for a set of 48 bi-allelic short insertion/deletion (indels) polymorphisms, validated as ancestry informative markers. The genotyping procedure was performed by three multiplex reactions as described elsewhere [16].

The 33 immune system gene SNPs were determined by allelic discrimination with Taqman 5'-nuclease assays. A total of 30 polymorphisms were genotyped with validated genotyping assays (Real Time PCR, Applied Biosystems, CA, USA). Three variants -174C > G (rs1800795), -863A > C (rs180030) and 874A > T (rs2430561) were genotyped by custom genotyping assay by design (Applied Biosystems, CA, USA). All assays were genotyped according to the manufacturer's recommended protocol.

Statistical analyses

Allele and genotype frequencies were estimated by gene counting. Deviation from Hardy-Weinberg equilibrium was assessed by Qui-square tests with Bonferroni correction. Haplotype frequencies and linkage disequilibrium were estimated with PHASE 2.1.1 [17]. Differences between malaria and control samples on age and ancestry were estimated by Mann–Whitney tests. The individual proportions of European, African and Amerindian genetic ancestry were estimated using the STRUCTURE software 2.3.3 assuming three parental populations (Europeans, Africans and Amerindians), and running with 200,000 burn-in period and 200,000 Markov Chain Monte Carlo repetitions after burning [18]. Poisson regressions were performed to assess the association between polymorphisms or haplotypes and age. The association between malaria cases and controls was performed using the STRAT software with 10,000 simulations [19]. STRAT utilizes the STRUCTURE output to test for association in the presence of population stratification based on individual ancestry information. Mann–Whitney, Qui-square and Poisson Regression tests were performed using the SPSS18.0 statistical package for Windows®. Statistical significance was defined as a two-tailed P-value < 0.05.

Results

The 33 SNPs investigated, their location in the gene and the allele frequencies observed in malaria cases and controls are shown in Additional file 1. The genotype distribution did not deviate significantly from Hardy-Weinberg equilibrium in both samples (Additional file 2).

Age and mean ancestry proportions of the subjects enrolled in the study are shown in Table 1. The control group was younger (32.8 ± 16.5 years) and presented a larger proportion of European ancestry (0.436 ± 0.13)

Table 1 Age and genetic ancestry of malaria and control subjects

Characteristics	Controls	Malaria	P value[a]
N	263	216	
Age (y)	32.8 ± 16.5	35.4 ± 15.3	0.003
Genetic Ancestry			
African	0.245 ± 0.10	0.246 ± 0.09	0.6
European	0.436 ± 0.13	0.412 ± 0.11	0.03
Native American	0.319 ± 0.11	0.342 ± 0.12	0.06

Values for age and genetic ancestry are expressed as mean ± SD.
[a] Mann–Whitney test p values.

than the malaria patients (35.4 ± 15.3 years; 0.412 ± 0.11 respectively). These differences were statistically significant between groups (p = 0.003 and p = 0.037, respectively). Due to this significant difference in age, a Poisson regression analysis using age as a covariate was performed. Only the polymorphisms with P <0.20 between malaria and control samples were chosen for further analyses (Additional file 3).

Because ancestry proportions differ between malaria patients and controls (Table 1), the association between 13 SNPs, chosen from the Poisson regression analyses, was performed using the STRAT software (Table 2). After population structure correction, only *IL1B* -5839C > T and *IL4R* 1902A > G polymorphisms were associated with malaria susceptibility. The *IL1B* -5839C and *IL4R* 1902A alleles are 8.2% and 6.2% respectively more frequent in malaria patients than in controls. Haplotype association tests adjusted for population stratification by the STRAT software are shown in Table 3. *IL12RB1* and *TNF* haplotypes were associated with malaria susceptibility. The *IL12RB1*AC (–1094/-641) haplotype is only present in the malaria sample whereas the *TNF* TATGG (–1031/-863/-857/-308/-238) haplotype is 2.5% more frequent in individuals with malaria.

Discussion

In a gene-based association study with 18 candidate genes for malaria susceptibility using 33 SNPs as genetic markers, this study demonstrated that *IL1B*, *IL4R*, *IL12RB1* and *TNF* genes were associated with susceptibility to *P. vivax* malaria in a population of Pará state, Brazil.

Cytokines are immunomodulatory proteins produced by a wide variety of cells, and with very complex activities. A functional cytokine network is a central element in the homeostasis of the immune response and its alteration may lead to an abnormal immune response. Hence, recent interest has focused upon genes regulating the cytokine expression; in particular on gene polymorphisms that may influence the levels of expression and therefore the overall immune response. Despite evidence demonstrating the importance of *IL1B*, *IL4R*, *IL12RB1* and *TNF* genes in *P. falciparum* malaria pathology, the influence of these variants in *P. vivax* infections is unknown.

Independent studies reported differences in immune system gene polymorphisms frequencies in distinct malaria-endemic regions [20]. The relevance of these polymorphisms in malaria infections could differ between distinct genetic background populations or etiologic agents, highlighting the importance of studies in different endemic regions. Due to the high admixed nature of the Brazilian population and its substructuring consequences in genetic association studies, this study dealt with this issue with extreme care. The population from Pará state has European, African and Amerindian ancestral groups [16,21], so that a structured population

Table 2 Structured population association test between malaria and control samples

Gene	SNP	dbSNP ID	Allele	Frequency (%)		P^2_{Qui}	P_{Strat}
				Control	Malaria		
IL1B	–5839C > T	rs1143629	C	46.2	54.4	0.01	**0.04**
IL4R	1902A > G	rs1801275	A	32.5	38.7	0.04	**0.02**
IL6	–174C > G	rs1800795	C	20.9	17.8	0.25	0.50
IL10	–592A > C	rs1800872	A	34.2	37.7	0.27	0.40
IL12B	458A > G	rs2546890	A	40.1	33.6	0.04	0.12
IL12RB1	–1094A > G	rs375947	G	23.4	20.9	0.36	0.19
	–641C > T	rs11575934	G	20.8	19.9	0.72	0.79
SP110	14622C > T	rs2114592	T	8.2	9.3	0.55	0.75
TNF	–1031C > T	rs1799964	C	24.0	21.9	0.44	0.10
	–238A > G	rs361525	A	7.4	6.1	0.44	0.37
	–857C > T	rs1799724	T	14.4	15.0	0.79	0.12
IFNG	874A > T	rs2430561	A	25.8	28.3	0.38	0.29
IFNGR1	–56 T > C	rs2234711	C	39.8	38.1	0.59	0.71

Table 3 Structured population association test for haplotypes association between malaria and control samples

Gene	Haplotype	Allele	Frequency (%)		P^2_{Qui}	P_{Strat}
			Control	Malaria		
IL1B	−5839/-31/-511	TTG	49.0	42.8	0.02	0.07
		TTA	0.2	0.2		
		TCG	2.9	0.9		
		TCA	1.5	1.6		
		CTG	0.2	1.5		
		CCA	46.2	53.0		
IL10	−592A/-819/-1082	CCA	36.3	39.3	0.17	0.57
		CCG	29.2	22.8		
		CTA	0.4	0.2		
		ATA	34.1	37.5		
		ATG	0	0.2		
IL12B	159/458/735	CGC	31.8	36.3	0.09	0.22
		CGT	1.4	4.0		
		CAC	0.7	0.2		
		CAT	2.7	2.4		
		AGC	11.6	10.1		
		AGT	14.1	15.8		
		AAC	0.2	0.5		
		AAT	37.5	30.7		
IL12RB1	−1094/-641	AT	76.7	77.8	0.04	**0.01**
		AC	0	1.4		
		GT	2.5	2.3		
		GC	20.8	18.5		
TNF	−1031/-863/-857/ -308/-238	TCCGG	57.8	61.4		
		TCCGA	1.6	0.2		
		TCCAG	6.6	6.7		
		TCTGG	11.1	9.8		
		TCTGA	0.5	0.2		
		TCTAG	0	0.2		
		TACGG	1.3	0.2	0.07	**0.01**
		TATGG	1.3	3.8		
		CCCGG	1.6	0.2		
		CCCGA	0.5	1.0		
		CCTGG	0.8	0.2		
		CACAA	16.3	15.7		
		CACGA	0.3	0.2		
		CATGG	0.3	0.2		
IFNGR1	−56 /611	TA	32.7	36.1	0.28	0.57
		TG	28.1	25.9		
		CA	38.0	37.7		
		CG	1.2	0.3		

association test to avoid genetic bias in the analysis was employed to provide reliable results for this specific population.

The IL-1β, IL-12 and the TNF together with IFN-γ are the major cytokines in pro-inflammatory Th1 immune response. IL-1β is predominantly secreted by monocytes and macrophages in initial immune response against infections [22-24] and helps to modulate the expression of IFN-γ and promote the polarization to Th17 immune response in certain circumstances [25,26]. IL-12 promotes IFN-γ production by T and natural killer (NK) cells and exerts its biological function through binding to the heteromeric interleukin 12 receptor (IL-12R) β1 and β2. The deficiency in IL-12R expression interferes in IL-12 functions and is associated with severe infection in humans [27,28]. TNF is produced by monocytes and macrophages and its role in malaria pathology was investigated due to reports of high levels of this cytokine in cerebral malaria patients [29].

IL1B gene was associated with *P. falciparum* malaria in African populations only [30,31]. The present study is the first to report the association of -5839C > T SNP promoter with *P. vivax* malaria susceptibility. The -5839C allele presented a higher frequency in malaria patients then in controls. Despite that, the function of this intronic SNP is not completely elucidated, variability of this important pro-inflammatory gene could represent an important factor in immune regulation. The association of haplotype -31C/-511A in *IL1B* gene promoter with severe malarial anaemia and circulating IL-1β low levels in children with *P. falciparum* malaria from Kenya have been shown recently [32], however, the *IL1B* -31C > T polymorphism was not associated with cerebral malaria in Thailand [33]. In the present study these two SNPs were not associated with vivax malaria, demonstrating a possible difference in the contribution of these polymorphisms to malaria pathology among populations and parasites. Complementary studies in *IL1B* gene and IL-1β levels are important to help understand how this gene influences malaria susceptibility and severity.

Recent work in Kenyan patients infected with *P. falciparum* demonstrated that *IL12RB1* rs4229774 and rs383483 polymorphisms were associated with protection against severe malarial anaemia and high parasitaemia levels, but not susceptibility [34]. The present study reports the association of *IL12RB1* -1094A/-641C haplotype with *P. vivax* malaria susceptibility. Despite the lower frequency in the study subjects, this haplotype is present only in the individuals with malaria, suggesting a possible influence in malaria response. The -641C allele leads to a missense variant (i.e. encodes a different amino acid) and can modify the receptor properties and interfere with IL-12 ligation and function. These results suggest that *IL12RB1* variants are important in malaria

susceptibility and severity. Further studies will be necessary to better understand the *IL12RB1* influence on susceptibility and severity in *P. vivax* malaria.

Polymorphisms in *TNF* gene promoter have been reported to be associated with symptoms and severity of *P. falciparum* malaria in different African and Asian populations [35-39]. The present work demonstrated that the TATGG (–1031/-863/-857/-308/-238) *TNF* haplotype is associated with *P. vivax* malaria in a Brazilian population. TNF is an important pro-inflammatory cytokine and the TATGG haplotype diverge in two alleles (–1031C and -308A) associated with TNF levels in vivax malaria infection in India [12]. In that work it was hypothesized that -1031C and -308A alleles are rare in Indian malaria patients due to a possible protective effect. The presence of -1031 T and -308 G alleles in TATGG haplotype could be an important factor in vivax malaria susceptibility.

Interleukin 4 receptor (IL-4R), together with IL-4 and IL-13 are important Th2 anti-inflammatory immune response modulators. IL-4R is the principal receptor of these interleukins, and when it is blocked IL-4 and IL-13 function is aborted preventing Th2 immune response modulation [40]. Only a few works investigated IL-4R variants influence on malaria infections. In the present investigation an association of 1902A > G SNP with malaria susceptibility was observed. The 1902 G allele create a missense variant and potentially can modify the receptor properties and interfere with IL-4 and IL-13 functions. It has been shown that the immune response via IL-4, IL4R and IL-13 pathway is important to prevent malaria infection in mice [41,42]. These studies demonstrated that knockout mice for *IL4* and *IL4R* genes have high resistance to malaria liver stage caused by sporozoites. The absence of modulation mediated by IL-4 to Th2 immune response, the Th1 response mediated by IFN-γ is maintained and promotes a rapid cellular response against sporozoites. The present study shows that 1902A allele is more frequent in malaria patients, although 1902 G presents a higher overall frequency in the study population, 1902A allele could influence the co-regulation between Th1 and Th2 immune response against malaria infections. New complementary studies will be necessary to elucidate the *IL4R* gene importance in vivax malaria infection.

IL4, *IL10*, *IL12B*, *IFNG* and *IFNGR1* genes polymorphisms were associated with symptoms and severity of *P. falciparum* malaria in studies from African and Asian populations [11]. In the present study, polymorphisms in these genes were not associated with susceptibility to malaria caused by *P. vivax*. Differences in malaria pathophysiology caused by these two species of parasites could be a possible explanation for the divergences reported. *Plasmodium falciparum* malaria presents a more acute form of infection reaching more than 50% of erythrocytes leading to cyto-adherence, organ damaged, severe malarial anaemia and cerebral malaria. Malaria caused by *P. vivax* is characterized by a long incubation period and milder initial symptoms. The parasite infects approximately 2 to 5% of erythrocytes, and can remain dormant in the liver as hypnozoites leading to subsequently relapses [43]. Studies have linked high levels of pro-inflammatory cytokines, such as TNF, IL-1β, IL-6 and IFN-γ in *P. falciparum* infections [44-47] and TNF, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10 e IL-12 cytokines with *P. vivax* infections [13,48-50]. These differences in cytokine profile and gene polymorphisms should reflect a distinct dynamic between the regulatory pathway of pro- and anti-inflammatory cytokines in *P. falciparum* and *P. vivax* malaria response, pathology and outcome.

Conclusions

Knowledge of the relationships between genetics, susceptibility to and severity of malaria infections is essential to identify subjects at higher risk and to develop specific preventive measures. This study showed that genetic polymorphisms of some interleukins involved in the immune response to *P. vivax* infection influence susceptibility to malaria. The present findings reinforce and increase our understanding about the role of the immune system in malaria susceptibility. However, to expand knowledge about the actions of the immune system in the pathophysiology of vivax malaria it is necessary to conduct population-based longitudinal studies.

Additional files

Additional file 1: Table S1. List of SNPs investigated in the present study. List of SNPs investigated, their location in the gene, pubmed database SNP identification, manufacturer's assay identification and allele frequencies in case and control groups.

Additional file 2: Table S2. Control and malaria group genotypic frequencies. Genotypic frequencies of all SNPs in case and control groups.

Additional file 3: Table S3. Poisson Regression for association between malaria and control samples controlling for age. Poisson Regression results for association between malaria and control samples controlling for age.

Abbreviations

Th: T helper; SNP: Single nucleotide polymorphism; TNF: Tumour necrosis factor; IFN-γ: Interferon gamma; IL: Interleukin; Indels: Insertion/deletion; NK: Natural killer; IL-12R: Interleukin 12 receptor; IL-4R: Interleukin 4 receptor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VAS designed the research project, carried out the laboratory assays, and wrote the manuscript; MGC participated in the study design, planning and data collection, revised the manuscript for important intellectual content and approved the version to be published; MDOO participated in the study

design, planning and data collection, performed the malaria diagnosis and revised the manuscript for important intellectual content and approved the version to be published; JMS participated in the study design, planning and data collection, performed the malaria diagnosis and revised the manuscript for important intellectual content and approved the version to be published; NPCS participated in the study design, planning and data collection, performed the malaria diagnosis and revised the manuscript for important intellectual content and approved the version to be published; AKCR participated in the study design, planning and data collection, revised the manuscript for important intellectual content and approved the version to be published; SMC-J participated in the study design, planning and performed the statistical analysis and approved the version to be published; SEBS genotyped the ancestry informative markers, revised the manuscript for important intellectual content, and approved the version to be published; MHH conceived the study, and participated in its design and coordination, analysed the data, and wrote the final version of the article. All authors read and approved the final manuscript.

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Additional Table 1. List of SNPs investigated in the present study

Gene	Location	SNP	dbSNP ID	Assay ID	Allele	Frequency	
						Control	Malaria
IL1B	intron	-5839C>T	rs1143629	C___1839945_1_	T	0.538	0.456
					C	0.456	0.544
	5' upstream	-31C>T	rs1143627	C___1839944_10	C	0.504	0.496
					T	0.556	0.444
	5' upstream	-511A>G	rs16944	C___1839943_10	G	0.521	0.451
					A	0.479	0.549
IL2	5' upstream	-330G>T	rs2069762	C__15859930_10	T	0.712	0.641
					G	0.288	0.359
IL4	5' upstream	-590C>T	rs2243250	C__16176216_10	C	0.587	0.530
					T	0.413	0.470
IL4R	missense	1902A>G	rs1801275	C___2351160_20	G	0.675	0.613
					A	0.325	0.387
IL6	5' upstream	-174C>G	rs1800795*		G	0.791	0.822
					C	0.209	0.178
IL8	5' upstream	-251A>T	rs4073	C__11748116_10	A	0.456	0.458
					T	0.544	0.542
IL10	5' upstream	-592A>C	rs1800872	C___1747363_10	A	0.342	0.377
					C	0.658	0.623
	5' upstream	-1082T>C	rs1800896	C___1747360_10	G	0.707	0.771
					A	0.293	0.229
	5' upstream	-819C>T	rs1800871	C___1747362_10	T	0.345	0.379
					C	0.655	0.621
IL12A	3' UTR	121G>A	rs568408	C___2423981_10	A	0.108	0.089
					G	0.892	0.911
IL12B	5' upstream	735T>C	rs7709212	C__31985592_10	T	0.440	0.469
					C	0.560	0.531
	intron	458A>G	rs2546890	C__15894458_10	A	0.401	0.336

					G	0.599	0.664
	3' UTR	159A>C	rs3212227	C___2084293_10	C	0.385	0.430
					A	0.615	0.570
IL12RB1	missense	-1094A>G	rs375947	C____795442_1_	A	0.766	0.791
					G	0.234	0.209
	missense	-641C>T	rs11575934	C__27529556_10	C	0.208	0.199
					T	0.792	0.801
SP110	intron	14622C>T	rs2114592	C__15816049_10	C	0.918	0.907
					T	0.082	0.093
	missense	1274C>T	rs3948464	C___2915061_10	C	0.880	0.863
					T	0.120	0.137
TNF	5' upstream	-308C>T	rs1800629	C___7514879_10	A	0.076	0.073
					G	0.924	0.927
	5' upstream	-1031C>T	rs1799964	C___7514871_10	C	0.240	0.219
					T	0.760	0.781
	5' upstream	-238A>G	rs361525	C___2215707_10	A	0.074	0.061
					G	0.926	0.939
	5' upstream	-863A>C	rs1800630*		A	0.210	0.202
					C	0.790	0.798
	5' upstream	-857C>T	rs1799724	C__11918223_10	T	0.144	0.150
					C	0.856	0.850
TNFRSF1A	intron	303A>G	rs4149622	C__32131594_10	A	0.857	0.832
					G	0.143	0.168
IFNG	intron	874A>T	rs2430561*		T	0.742	0.717
					A	0.258	0.283
IFNGR1	5' upstream	611C>T	rs1327474	C___2523634_10	G	0.292	0.261
					A	0.708	0.739
	5' UTR	-56T>C	rs2234711	C__11693991_10	T	0.602	0.619
					C	0.398	0.381
VDR	missense	FokI	rs10735810	C__12060045_20	C	0.651	0.645

					T	0.349	0.355
	synonymous	TaqI	rs731236	C___2404008_10	T	0.729	0.701
					C	0.271	0.299
	intron	BsmI	rs1544410	C___8716062_10	G	0.719	0.726
					A	0.281	0.274
PTPN22	missense	R630W	rs2476601	C__16021387_20	G	0.949	0.951
					A	0.051	0.049
P2X7	missense	1513T>G	rs3751143	C__27495274_10	T	0.797	0.844
					G	0.203	0.156

*Custom assay

Additional Table 2. Control and malaria group genotypic frequencies

Gene	dbSNP ID	N	Genotypes		
IL1B					
-5839C>T	rs1143629		TT	TC	CC
Control		263	83 (31.5)	117 (44.5)	63 (24.0)
Malaria		216	48 (22.2)	101 (46.8)	67 (31.0)
-31C>T	rs1143627		CC	CT	TT
Control		263	80 (30.4)	105 (39.9)	78 (29.7)
Malaria		215	72 (33.5)	95 (44.2)	48 (22.3)
-511A>G	rs16944		GG	GA	AA
Control		261	80 (30.7)	112 (42.9)	69 (26.4)
Malaria		214	50 (23.4)	93 (43.5)	71 (33.1)
IL2					
-330G>T	rs2069762		TT	GT	GG
Control		262	130 (49.6)	113 (43.1)	19 (7.3)
Malaria		216	84 (38.9)	109 (50.5)	23 (10.6)
IL4					
-590C>T	rs2243250		CC	CT	TT
Control		263	88 (33.5)	133 (50.5)	42 (16.0)
Malaria		216	58 (26.9)	113 (52.3)	45 (20.8)
IL4R					
1902A>G	rs1801275		GG	GA	AA
Control		263	131 (49.8)	93 (35.4)	39 (14.8)
Malaria		216	78 (36.1)	109 (50.5)	29 (13.4)
IL6					
-174C>G	rs1800795		GG	GC	CC
Control		263	162 (61.6)	92 (35.0)	9 (3.4)
Malaria		216	147 (68.1)	61 (28.2)	8 (3.7)
IL8					
-251A>T	rs4073		AA	AT	TT
Control		263	56 (21.3)	128 (48.7)	79 (30.0)
Malaria		212	43 (20.3)	108 (50.9)	61 (28.8)
IL10					
-592A>C	rs1800872		AA	AC	CC
Control		263	35 (13.3)	110 (41.8)	118 (44.9)
Malaria		216	28 (13.0)	107 (49.5)	81 (37.5)
-1082T>C	rs1800896		GG	GA	AA
Control		263	133 (50.6)	106 (40.3)	24 (9.1)
Malaria		216	128 (59.3)	77 (35.6)	11 (5.1)
-819C>T	rs1800871		TT	TC	CC
Control		262	36 (13.7)	109 (41.6)	117 (44.7)
Malaria		215	29 (13.5)	105 (48.8)	81 (37.7)
IL12A					
121G>A	rs568408		AA	AG	GG
Control		223	5 (2.2)	38 (17.0)	180 (80.8)
Malaria		213	3 (1.4)	32 (15.0)	178 (83.6)
IL12B					
735T>C	rs7709212		TT	TC	CC
Control		226	70 (31.0)	113 (50.0)	43 (19.0)
Malaria		213	66 (31.0)	94 (44.1)	53 (24.9)
458A>G	rs2546890		AA	AG	GG
Control		237	49 (20.7)	92 (38.8)	96 (40.5)
Malaria		214	28 (13.1)	88 (41.1)	98 (45.8)
159A>C	rs3212227		AA	CA	AA
Control		260	49 (18.8)	102 (39.2)	109 (42.0)
Malaria		213	42 (19.7)	99 (46.5)	72 (33.8)
IL12RB1					

-1094A>G	rs375947		AA	AG	GG
Control		263	160 (60.8)	83 (31.6)	20 (7.6)
Malaria		215	132 (61.4)	76 (35.3)	7 (3.3)
-641C>T	rs11575934		GG	GA	AA
Control		262	17 (6.5)	75 (28.6)	170 (64.9)
Malaria		214	6 (2.8)	73 (34.1)	135 (63.1)
SP110					
14622C>T	rs2114592		CC	CT	TT
Control		262	221 (84.4)	39 (14.8)	2 (0.8)
Malaria		215	176 (81.9)	38 (17.6)	1 (0.5)
1274C>T	rs3948464		CC	CT	TT
Control		263	201 (76.4)	61 (23.2)	1 (0.4)
Malaria		215	160 (74.1)	53 (24.5)	3 (1.4)
TNF					
-308C>T	rs1800629		AA	AG	GG
Control		263	2 (0.8)	36 (13.7)	225 (85.5)
Malaria		213	1 (0.5)	29 (13.6)	183 (85.9)
-1031C>T	rs1799964		CC	CT	TT
Control		263	12 (4.6)	102 (38.8)	149 (56.6)
Malaria		215	9 (4.2)	76 (35.3)	130 (60.5)
-238A>G	rs361525		AA	AG	GG
Control		224	3 (1.3)	27 (12.1)	194 (86.6)
Malaria		214	3 (1.4)	20 (9.3)	191 (89.3)
-863A>C	rs1800630		AA	AC	CC
Control		252	22 (8.7)	62 (24.6)	168 (66.7)
Malaria		213	21 (9.9)	44 (20.7)	148 (69.5)
-857C>T	rs1799724		TT	TC	CC
Control		205	8 (3.9)	43 (21.0)	154 (75.1)
Malaria		213	6 (2.8)	52 (24.4)	155 (72.8)
TNFRSF1A					
303A>G	rs4149622		AA	AG	GG
Control		263	193 (73.4)	65 (24.7)	5 (1.9)
Malaria		214	146 (68.2)	64 (29.9)	4 (1.9)
IFNG					
874A>T	rs2430561		TT	TA	AA
Control		262	144 (55.0)	101 (38.5)	17 (6.5)
Malaria		214	111 (51.9)	85 (39.7)	18 (8.4)
IFNGR1					
-611(C>T)	rs1327474		GG	GA	AA
Control		209	21 (10.0)	80 (38.3)	108 (51.7)
Malaria		213	15 (7.0)	81 (38.1)	117 (54.9)
-56T>C	rs2234711		TT	TC	CC
Control		250	99 (39.6)	103 (41.2)	48 (19.2)
Malaria		214	82 (38.3)	101 (47.2)	31 (14.5)
VDR					
FokI	rs10735810		CC	CT	TT
Control		259	116 (44.8)	105 (40.5)	38 (14.7)
Malaria		214	90 (42.1)	96 (44.8)	28 (13.1)
TaqI	rs731236		TT	TC	CC
Control		262	146 (55.7)	90 (34.4)	26 (9.9)
Malaria		179	84 (46.9)	83 (46.4)	12 (6.7)
BsmI	rs1544410		GG	GA	AA
Control		263	139 (52.9)	100 (38.0)	24 (9.1)
Malaria		210	107 (51.0)	91 (43.3)	12 (5.7)
PTPN22					
R630W	rs2476601		GG	GA	AA
Control		263	236 (89.7)	27 (10.3)	0
Malaria		215	21 (9.8)	194 (90.2)	0

P2X7					
1513T>G	rs3751143		TT	TG	GG
Control		263	164 (62.4)	91 (34.6)	8 (3.0)
Malaria		211	148 (70.1)	60 (28.5)	3 (1.4)

Additional Table 3. Poisson Regression for association between malaria and control samples controlling for age

Gene	SNP	dbSNP ID	Risk Genotype	P value
IL1B	-5839C>T	rs1143629	TC	**0.02**
			CC	**0.08**
	-31C>T	rs1143627	CT	0.25
			TT	0.41
	-511A>G	rs16944	GA	0.80
			AA	0.75
IL2	-330G>T	rs2069762	GT	0.26
			TT	0.30
IL4	-590C>T	rs2243250	CT	0.95
			TT	0.87
IL4R	1902A>G	rs1801275	AG	**0.009**
			GG	0.31
IL6	-174C>G	rs1800795	GC	**0.01**
			CC	0.34
IL8	-251A>T	rs4073	AT	0.72
			TT	0.59
IL10	-592A>C	rs1800872	CA	0.42
			CC	**0.15**
	-1082T>C	rs1800896	GA	0.32
			GG	0.61
	-819C>T	rs1800871	CT	0.25
			TT	0.73
IL12A	121G>A	rs568408	AG	0.93
			GG	0.98
IL12B	735T>C	rs7709212	TC	0.30
			TT	0.31
	458A>G	rs2546890	AG	**0.01**
			GG	**0.02**
	159A>C	rs3212227	AC	0.22
			AA	0.67
IL12RB1	-1094A>G	rs375947	GA	**0.009**
			GG	**0.003**
	-641C>T	rs11575934	GA	0.56
			AA	**0.07**
SP110	14622C>T	rs2114592	CT	**0.13**
			CC	**0.15**
	1274C>T	rs3948464	CT	0.30
			CC	0.32
TNF	-308C>T	rs1800629	AG	0.84
			AA	0.80
	-1031C>T	rs1799964	CT	0.85
			TT	**0.13**
	-238A>G	rs361525	AG	**0.001**
			GG	**0.001**
	-863A>C	rs1800630	AC	0.82
			CC	0.23
	-857C>T	rs1799724	TC	0.76

			TT	**0.05**
TNFRSF1A	303A>G	rs4149622	AG	0.71
			GG	0.73
IFNG	874A>T	rs2430561	TA	**0.18**
			TT	**0.13**
IFNGR1	-611C>T	rs1327474	GA	0.85
			AA	0.60
	-56T>C	rs2234711	CT	0.80
			CC	**0.02**
VDR	FokI	rs10735810	CT	0.92
			CC	0.34
	TaqI	rs731236	TC	0.46
			CC	0.86
	BsmI	rs1544410	AG	0.58
			AA	0.30
PTPN22	R630W	rs2476601	GA	0.23
P2X7	1513	rs3751143	TG	0.54
			GG	0.23

*Age is included as a covariate in Poisson regression

CAPÍTULO 4

ROLE OF IL6, IL12B AND VDR GENE POLYMORPHISMS IN *PLASMODIUM VIVAX* MALARIA PARASITEMIA AND GAMETOCYTEMIA LEVELS IN AN AMAZONIAN BRAZILIAN POPULATION

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Title: Role of *IL6*, *IL12B and VDR* gene polymorphisms in *Plasmodium vivax* malaria parasitemia and gametocytemia levels in an Amazonian Brazilian population

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Abstract

Objective: To investigate the influence of *IL6*, *IL12B* and *VDR* single nucleotide polymorphisms (SNPs) in *P. vivax* infection parasitemia and gametocytemia levels in a Brazilian amazonian population.

Methods: A total of 167 malaria patients infected by *P. vivax* have parasitemia and gametocytemia levels estimated before treatment. Patients were genotyped with *IL6* -174C>G, *IL12B* 735T>C, 458A>G, 159A>C, and *VDR* FokI, TaqI, BsmI SNPs by Taqman 5' nuclease assays. A General Linear Model analysis of covariance with age, gender, exposure period and infection history was performed to investigate the association of genotypes with parasitemia and gametocytemia levels.

Results: Higher parasitemia levels were observed in *IL6* -174C ($p = 0.02$) whereas *IL12B* CGT haplotype carriers presented lower parasitemia levels ($p = 0.008$). *VDR* TaqIC/BsmIA haplotype carriers showed higher gametocyte levels than non-carriers ($p = 0.013$).

Conclusion: The present study suggests that *IL6*, *IL12* and *VDR* SNPs might influence parasitemia and gametocytemia clearance in *P. vivax* infections, and highlights its potential role in malaria immune response in an Amazonian population.

Keywords: Malaria, *P. vivax*, Parasitemia, *IL6*, *IL12B*, *VDR*

1. Introduction

Malaria is a major cause of human mortality worldwide and is considered to be one of the strongest known forces of evolutionary selection in the recent history of the human genome [1]. Host genetic defense mechanisms are likely to have evolved to resist malaria infection in regions where the parasites have been historically prevalent. Together with parasite virulence phenotypes and host's immune response, the level of parasitemia, has been considered one of the most important conditioning factors of malaria susceptibility and severity

Half the world's population is estimated to be at risk for malaria caused by *Plasmodium vivax*, owing to this parasite's unique potential for lengthy remission and tolerance at cooler climates than those preferred by strictly tropical *Plasmodium* species [2]. *P. vivax* infections has peculiar biological features such as merozoites predilection or reticulocytes as host cells, the early presence of round gametocytes in the peripheral blood, the circulation at all blood-stage developmental forms in the peripheral blood, and most important, the development of dormant hypnozoite forms in the liver that cause subsequent relapses [3]. Malaria induces an early pro-inflammatory immune response by a set of T helper (Th) 1 cells stimulating the release of cytokines and phagocyte cells, which have an important role in parasite clearance by activating immune effectors mechanisms. A later anti-inflammatory response mediated by a set of Th2 cells and monocytes is required to control malaria preventing organ damages and more severe symptoms [4].

Interleukin(IL)s 6 and 12 are secreted by endothelial and antigens presenting cells in response to *P. vivax* infection. IL-12 levels were inversely correlated with parasitemia in *P. falciparum* infections and polymorphisms in *IL12* gene were associated with infection severity [5-7]. IL-6 levels are elevated in *P. vivax* infection and it is suggested to be involved in parasite clearance [8,9], however *IL6* genetic variants were poorly investigated in malaria infections.

The active form of vitamin D ($1,25(OH)_2D_3$), is an immune modulator and its interaction with the vitamin D receptor (VDR) influences both innate and adaptive immunity in response against intracellular pathogens. *VDR* gene polymorphisms were associated with mycobacteria and viral infections [10], but its relation to malaria has not been assessed so far.

The immune response mechanisms related to *P. vivax* infection clearance are poorly understood. The study of host immune system polymorphisms might be a valuable tool for risk assessment and progression of infectious diseases, therefore the present study addressed the role of *IL6*, *IL12B* and *VDR* single nucleotide polymorphisms (SNPs) in *P. vivax* parasitemia and gametocytemia levels in a Brazilian population from the Amazonian region.

2. Methods

2.1. Study population

A total of 167 patients who were born in Pará state in the Brazilian Amazonian region were diagnosed with *P. vivax* malaria at the Evandro Chagas Institute, Belém, Pará between 2002-2009. Immediately after diagnosis, the patients had their blood collected for parasite density estimates and DNA analyses. All patients received the standard 1500 mg of chloroquine associated with 210 mg of primaquine treatment in seven days. All subjects provided their written informed consent to participate in this study. The Ethics Committees of the Evandro Chagas Institute and Federal University of Pará approved the study protocol.

2.2. Malaria Diagnoses and Parasitemia Estimates

Patients were diagnosed with *P. vivax* malaria by thick blood smear, as recommended by the Brazilian Ministry of Health [11]. Asexual and sexual (gametocyte) forms density per μl of blood was estimated by counting the number of parasites per 100 fields and double-checked blindly by two expert microscopists.

2.3. Genotyping

Genomic DNA was extracted by standard procedures. The SNPs were determined by allelic discrimination with Taqman 5'-nuclease assays according to the manufacturer's recommended protocol. The *IL12B* 735T>C (rs7709212), 458A>G (rs2546890), 159A>C (rs3212227) SNPs, and *VDR* FokI (rs10735810), TaqI (rs731236), BsmI (rs1544410) SNPs were genotyped with validated genotyping assays (Applied Biosystems, CA, USA). The *IL6* -174C>G (rs1800795) SNP, were genotyped by custom genotyping assay by design (Applied Biosystems, CA, USA). All patients were also genotyped for ancestry informative markers as previously described [12].

2.4. Statistical analyses

Allele and genotype frequencies were estimated by gene counting. Deviation from Hardy-Weinberg equilibrium was assessed by Qui-square tests with Bonferroni correction. Haplotype frequencies and linkage disequilibrium were estimated with PHASE 2.1.1. The individual proportions of European, African and Amerindian ancestry were estimated using the STRUCTURE software 2.3.3 assuming three parental populations: Europeans, Africans and Amerindians [12]. General Linear Model (GLM) analyses of covariance were used to assess the association of genotypes and parasite levels. Potential covariates to be entered in models were defined based on conceptual analyses of the literature and/or by means of a statistical definition (association with the study factor and with the outcome at $P \leq 0.15$). The GLM analyses were performed using the SPSS18.0 statistical package for Windows®. Statistical significance was defined as a two-tailed P-value < 0.05.

3. Results

Malaria patients were aged between 12 and 88 years (36.0 ± 15.6 years), 67.0% were males and presented 0.241 African, 0.417 European and 0.342 Native American mean genetic ancestry proportions (Table 1). Genotype distributions did not deviate significantly from Hardy-Weinberg equilibrium. Allele, genotype and haplotype frequencies are show in Supplemental tables 1 and 2.

Parasitemia levels ranged from 50 and 75,000 parasites/μL (8,606.5 ± 10,187.4) and gametocytemia from 15 and 4,500 gametocytes/μL (295.2 ± 622.3). In the study population, 68% of the individuals presented malaria for the first time, 12.2% were infected for the second time and 19.8% of the investigated subjects were infected for more than two times. The exposure period in malaria endemic regions was variable, 53.8% stayed up to 25 days, 26.1% more than 25 days and 20.1% were residents (Table 1). Genetic ancestry estimates did not differ among genotype and haplotype distributions or parasitemia and gametocytemia levels ($p > 0.05$).

After adjustment for age, gender, exposure time and infection history, *IL6* -174C carriers presented higher parasitemia levels when compared to -174GG homozygotes (6.254.1 versus 3.529.8 parasites/μL, $p = 0.02$) (Table 2). Lower parasitemia levels were observed in *IL12B* 159C/458G/735T haplotype carriers in relation to other haplotypes ($p = 0.008$) (Table 2). *IL6* and *IL12* SNPs were not associated with gametocytemia levels.

VDR TaqI and BsmI CA haplotype carriers showed higher gametocyte levels than non-carriers (225.5 versus 113.3 gametocytes/μL p = 0.013) (Table 2). This haplotype was not associated with parasitemia levels. FokI SNP is not in linkage disequilibrium with the other two SNPs and was not associated with parasitemia or gametocytemia levels. (Table 2).

4. Discussion

Although there are several studies that reports genetic susceptibility to severe malaria in African populations, much less information is available about how variation in immune response genes influence parasitemia density in other populations out of Africa. The main finding of the present study was that *IL6*, *IL12B* and *VDR* genes are independent predictors of parasitemia and gametocytemia densities in *P. vivax* infection in a population of Pará state, Brazil.

CD4+ T cells and macrophages develop enhanced microbicidal activity by cytokines secretion and neutrophils, eosinophils and basophils recruitment to assist innate immunity. In addition, they help B cells to produce antibodies and license dendritic cells to modulate CD8+ cytotoxic T lymphocyte responses in adaptive immunity. Depending on cytokine immunomodulatory signals, naïve CD4+ T cells can develop distinct functional CD4+ T cell subsets. Cell differentiation associated with the production of Th1 signature cytokine interferon-γ (IFN-γ) is required for efficient immune responses against intracellular pathogens. [4].

IL-12 is a cytokine released by monocytes, macrophages, B cells, and dendritic cells. These interleukin promotes IFN-γ production by T and natural killer (NK) cells as part of the host immune response to invading pathogens. IL-12 is a heterodimer composed of IL-12p35 and IL-12p40 subunits, encoded by *IL12A* and *IL12B* genes located on chromosomes 3p12-q13.2 and 5q31-33, respectively, and exerts its biological function through binding to the heteromeric interleukin 12 receptor (IL-12R) β1 and β2 [13]. The present study showed *IL12B* 159C/458G/735T haplotype association with low parasitemia levels in *P. vivax* infection.

Previous examination of IL12B polymorphisms has shown conflicting results. Several studies reported association of The 159A>C with *P. falciparum* severity [5,6,14]. However, no association with severity was observed in costal Kenia and in Thais [7,14-

16]. In the present study carriers of *IL12B* CGT haplotype seems to have lower parasitemia density as compared to other haplotype carriers. The discrepancy among studies might be due to different study designs, ethnic background of the population and intrinsic differences between *P. falciparum* and *P.vivax* infections.

IL-6 is a pleiotropic cytokine which exerts pro- and anti-inflammatory activities by two different pathways. The IL-6 classic signaling through specificity-defining membrane IL-6 receptor (IL-6R) expressed in hepatocytes and immune system cells is correlated with regenerative and anti-inflammatory activities. Moreover, in a *trans-signaling* process IL-6 is able to use the soluble IL-6 receptor (sIL-6R) and binds to a ubiquitously expressed membrane GP130 protein exerting primarily pro-inflammatory activities [17]. Circulating IL-6 levels are elevated in *P. vivax* and *P. falciparum* infections, and are associated with severe malaria in humans [8]. sIL-6R serum levels were also correlated with parasite clearance time in *P. falciparum* infection [9]. The present investigation reports the role of *IL6* 5' upstream -174C>G SNP in *P. vivax* parasitemia counts. This SNP was previously associated with *IL6* gene expression and IL-6 levels in healthy volunteers. *IL6* -174GG homozygotes showed almost twice higher levels of circulating IL-6 than CC homozygotes suggesting a suppressive effect of C allele in IL-6 production [18]. In the present study -174C carriers presented higher mean parasitemia counts as compared to GG homozygotes. Lower levels of circulating IL-6 due to the presence of the C allele could be potentially related to defective parasite clearance.

The $1,25(OH)_2D_3$/VDR interactions modulate immune functions as cathelicidin and antimicrobial peptides expression in monocytes and macrophages. $1,25(OH)_2D_3$ also reduce the expression of pro inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IFN-γ, IL-2 in immune cells, and can induce the expression of anti-inflammatory cytokines IL-4 and IL-10 [19]. Our study reports that TaqIC/BsmIA haplotype carriers presented higher gametocytemia densities compared to other haplotype carriers. Given that similar results were observed in individuals with tuberculosis [10]. This haplotype could be a marker of susceptibility to intracellular pathogens. Additional studies are required to confirm this hypothesis.

The exactly mechanisms by which IL-6, IL-12 and Vitamin D influence and act in parasite clearance is difficult to determine, because interleukins create a complex net to regulate immune system processes. IL-6 regulates the expression of diverse molecules and

cytokines and also is regulated by the expression of different cytokines as IL-1β, TNF-α, IL-4, and IL-10. Furthermore, IL-6 and IL-12 families share intra and intercytokines protein subunits and receptors [13,17]. Vitamin D also can inhibit Th1 and induce Th2 responses [19]. The extensive cytokine plasticity and "cross-talking" create a complex scenario to understand the biological function of these molecules in infection control. Genetic variants in immune modulator genes could point to new insights about different malaria infection aspects. Our results suggest an important role for *IL6*, *IL12* and *VDR* gene polymorphism in pro inflammatory response in parasitemia and gametocytemia clearance in *P. vivax* infection. Future functional studies are warranted to unveil the complex *P. vivax* immune response.

5. Conclusions

Cytokines are major players in immune response to malaria and to understand how genetic variants influence host defense mechanisms may be an important issue for infection prevention. The present investigation showed that parasitemia and gametocytemia levels in *P. vivax* malaria might be modulated by *IL6*, *IL12* and *VDR* SNPs, and highlights the potential role of these cytokines in infection response. Future functional studies are warranted to better understand the role of these genes in *P.vivax* infections.

Conflict of interests: The authors have no conflict of interest to declare.

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Table 1. Study group characteristics

Characteristics	**Malaria Patients**
N	167
Age	36.0 (15.6)
Gender (male %)	67.0
Exposure period (%)	
1 to 25 days	53.8
more than 25 days	26.1
Residant	20.1
Infections (%)	
First	68.0
Two	12.2
More than 2	19.8
Parasitemia (Parasites/μL)	8,606.5 (10,187.4)
Gametocytemia (Gametocytes/ μL)	295.2 (622.3)
Genetic Ancestry	
African	0.241 (0.09)
European	0.417 (0.11)
Native American	0.342 (0.12)

Values for age, parasitemia, gametocytemia and genetic ancestry are expressed as mean ± SD

Table 2. Parasitemia and gametocytemia levels according to *IL6, IL12B* and *VDR* genotypes and haplotypes

Gene	SNP	N	Parasitemia	P	N	Gametocytemia	P
IL6	-174C>G						
	GG	114	3,529.8 (2,530.1:4,924.6)	**0.02**	47	130.6 (72.4:235.6)	0.1
	GC+CC	53	6,254.1 (4,052.1:9,652.8)		25	201.7 (104.3:390.3)	
IL12B	(159/458/735)						
	CGC/CGC	26	6,002.9 (3,374.5:10,689.3)		12	117.9 (54.0:257.5)	
	CGC/others	36	5,255.3 (3,105.7:8,892.8)		20	117.4 (55.7:247.9)	
	CGT carriers	13	1,044.2 (461.3:2,363.7)	**0.008**	1	39.7 (3.5:456.2)	0.2
	AAT carriers	78	4,491.8 (3,087.1:6,535.5)		34	168.0 (85.3:330.6)	
	Others	11	4,385.2 (1,870.6:10,290.7)		4	377.3 (110.2:1292.1)	
VDR	(TaqI/ BsmI)						
	CA carriers	80	3,659.2 (2,475.0:5,410.0)	0.2	35	225.7 (122.6:414.9)	**0.013**
	Non-carriers	76	4,717.3 (3,184.3:6,988.4)		35	113.3 (65.0:197.6)	
	FokI						
	TT	25	4,675.1 (2,514.9:8,690.6)		14	166.8 (73.4:378.8)	
	TC	71	3,944.2 (2,643.9:5,884.0)	0.8	30	174.3 (90.7:335.3)	0.6
	CC	69	4,546.0 (3,081.0:6,714.3)		27	129.7 (67.2:250.1)	

Parasitemia and gametocytemia levels were adjusted in the model for age, gender, exposure period and infections history.

Values are expressed as geometric mean and 95% confidence interval.

IL6 -174C>G genotypes were grouped because CC genotype were found in only in three subjects.

IL12B parasitemia: CGT carriers vs CGC/CGC $p = 0.021$, CGT carriers vs CGC/others $p = 0.014$, CGT carriers vs AAT carriers $p = 0.008$, CGT carriers vs others $p = 0.005$. Others combinations presents $p > 0.05$. All pairwise analysis were performed with bonferroni correction.

Supplemental table 1. *IL6*, *IL12* and *VDR* genotypic and allelic frequency

Gene		**dbSNP ID**	**N**	**Genotypes**			**Alleles**	
IL6	-174C>G	rs1800795	167	GG	GC	CC	G	C
				114 (68.2)	50 (30.0)	3 (1.8)	83.3	16.7
IL12B	159A>C	rs3212227	165	CC	CA	AA	C	A
				36 (21.8)	72 (43.6)	57 (34.6)	43.6	56.4
	458A>G	rs2546890	165	AA	AG	GG	A	G
				27 (16.3)	61(37.0)	77 (46.7)	34.8	65.2
	735T>C	rs7709212	164	TT	TC	CC	T	C
				43 (26.2)	67 (40.8)	54 (33.0)	46.6	53.4
VDR	FokI	rs10735810	165	CC	CT	TT	C	T
				25 (15.2)	71 (43.0)	69 (41.8)	36.7	63.3
	TaqI	rs731236	157	TT	TC	CC	T	C
				71 (45.3)	75 (47.7)	11 (7.0)	69.1	30.9
	BsmI	rs1544410	164	GG	GA	AA	G	A
				77 (47.0)	76 (46.3)	11 (6.7)	70.1	29.9

Supplemental table 2. *IL12B* and *VDR* haplotype frequency

Gene	**Haplotype**			**Alelos**	**N**	**Frequency (%)**
IL12B	735T>C	458A>G	159A>C			
	C	G	C	1	154	36.3
	C	G	T	2	17	4
	C	A	C	3	1	0.2
	C	A	T	4	10	2.4
	A	G	C	5	43	10
	A	G	T	6	67	15.8
	A	A	C	7	2	0.5
	A	A	T	8	130	30.7
VDR	TaqI	BsmI				
	C	A		1	98	27.4
	C	G		2	9	2.5
	T	A		3	9	2.5
	T	G		4	242	67.6

CAPÍTULO 5

PLASMODIUM VIVAX MALARIA SEVERITY IN BRAZILIAN AMAZONIAN POPULATION IS ASSOCIATED WITH IL6 AND IL10 GENE POLYMORPHISMS

Manuscrito em preparação para ser submetido à revista Infections and Immunity

Title: *Plasmodium vivax* malaria severity in Brazilian Amazonian population is associated with *IL6 and IL10* gene polymorphisms

Running Title: *IL6* and *IL10* SNPs and *P. vivax* malaria severity

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ABSTRACT

Plasmodium vivax infection is an important cause of morbidity worldwide and severe cases are becoming more common in the last years. The influence of Interleukin gene polymorphisms in immune response and severity in such infections is still poorly understood. The present study investigates the association of *IL4* -590C>T, *IL4R* 1902A>G, *IL6* -174C>G, *IL10* -1082T>C, -819C>T, -592A>C polymorphisms in *P. vivax* malaria severity in an Amazonian population. A total of 147 symptomatic *P. vivax* malaria patients who were born in Pará state, Brazil were evaluated. A General Linear Model analysis of covariance with age, gender, exposure period, infection history, parasitemia levels and genetic ancestry as covariates, was performed to investigate the association between interleukin polymorphisms and a clinical malaria severity index obtained by Principal Component Analysis of main malaria symptoms. *IL4* -590C>T and *IL4R* 1902A>G were not associated with malaria severity. *IL6* -174C carriers and homozygotes for *IL10* GCC (-1082/-819/-592) haplotype presented a higher clinical index value ($p = 0.001$ and $p = 0.03$ respectively). These results suggest that *IL6* and *IL10* polymorphisms are important factors in overall clinical symptom intensity in uncomplicated *P. vivax* malaria in Brazilians.

INTRODUCTION

Malaria is a major parasitic disease with half the world's population living in endemic areas (1). Traditionally treated as a milder infection, *P. vivax* malaria severe cases are becoming more common presenting symptoms as hepatic dysfunction, acute respiratory distress, renal failure, splenic rupture, severe anemia and cerebral malaria (3-7). Mechanisms leading to malaria milder or severe symptoms are not fully understood, but immune system failure to control overwhelming parasite replication or immunopathology resulting from excessive inflammation are considered to be contributing factors (8). Malaria infection triggers a pro-inflammatory response by a set of T helper (Th) 1 cells stimulating the release of cytokines and phagocytes cells, which have an important role in parasite clearance. A later anti-inflammatory response mediated by a set of Th2 cells and monocytes inhibit pro-inflammatory response, controlling malaria infection, and preventing more severe symptoms. Either an extreme pro-inflammatory response or a weak anti-inflammatory response can lead to symptom severity increase (8-11).

Immunomodulatory proteins as interleukin (IL) 4, IL-6 and IL-10 related to pro- and anti-inflammatory balance in the immune response can influence malaria infection and outcome. Interleukin 4 is an important inductor of Th2 cell differentiation and immunoglobulin E (IgE) synthesis. IL-4R is the IL-4 and IL-13 major receptor, and when it is blocked these cytokines function are aborted preventing anti-inflammatory immune response (12). *IL4* gene variants were associated with *P. falciparum* infection susceptibility and severity (13-16). Recently, an *IL4R* single nucleotide polymorphism (SNP) was associated with *P. vivax* malaria susceptibility in an Amazonian population (17).

IL-10 is a potent immunoregulatory cytokine with major anti-inflammatory effect by suppressing dendritic cells and macrophage functions. This interleukin also suppress pro-inflammatory cytokines and enhances the expression of their antagonists (18,19). *IL10* gene variants were associated with IL-10 production and protection against *P. falciparum* severity (20,21). However in a recent study, *IL10* -1082C>T SNP was not associated with IL-10 circulating levels or parasitemia in *P. vivax* infections (22).

IL-6 is a pleiotropic cytokine that utilizes two different pathways to exert pro- and anti-inflammatory activities. IL-6 levels are associated with malaria severity in humans and

animal models (23-25), however genetic variants in *IL6* gene were poorly investigated in *P. vivax* infections.

The role of Immune response in *P. vivax* infection severity is not completely understood; therefore the present study investigates *IL4, IL4R, IL6* and *IL10* gene variants and their association with *P. vivax* malaria severity in an Amazonian population.

MATERIAL AND METHODS

Study population. The study cohort consisted of 147 symptomatic malaria patients who were born in Pará state in the Brazilian Amazonian region. Sample collection and procedures for individual ancestry determination were performed as previously described (17).

All patients were clinical examined by a physician and symptoms as fever, headache, chills, myalgia, arthralgia, back pain, abdominal pain, asthenia, dizziness, dyspnea, cough, nausea, vomiting and diarrhea were evaluated as numerical scores from 0 to 4 (absent, mild, moderate, severe and very severe, respectively). After clinical assessment and diagnosis all patients received the standard treatment 1500 mg of chloroquine associated with 210 mg of primaquine in seven days. All subjects provided their written informed consent to participate in this study. The Ethics Committees of the Evandro Chagas Institute and Federal University of Pará approved the study protocol.

Genotyping. Genomic DNA was extracted from peripheral blood leukocytes subjects using proteinase K digestion and standard phenol-chloroform procedures (26). Six immune system SNPs were determined by allelic discrimination with Taqman 5'-nuclease assays according to the manufacturer's recommended protocol. *IL4* -590C>T (rs2243250), *IL4R* 1902A>G (rs1801275), and *IL10* -592A>C (rs1800872), -819C>T (rs1800871) and -1082T>C (rs1800896) SNPs were genotyped with validated Real Time PCR genotyping assays (Applied Biosystems, CA, USA). The *IL6* -174C>G (rs1800795) SNP, were genotyped by custom genotyping assay by design (Applied Biosystems, CA, USA).

Statistical analyses. Allele and genotype frequencies were estimated by gene counting. Deviation from Hardy-Weinberg equilibrium was assessed by Qui-square tests with Bonferroni correction. Haplotype frequencies and linkage disequilibrium were estimated with PHASE 2.1.1. The individual proportions of European, African and

Amerindian genetic ancestry were estimated using the STRUCTURE software 2.3.3 assuming three parental populations (Europeans, Africans and Amerindians), and running with 200,000 burn-in period and 200,000 Markov Chain Monte Carlo repetitions after burning (27). A Principal Component Analysis was performed to aggregate symptom numerical scores into a clinical index that represents the overall intensity of malaria symptoms (28,29). A General Linear Model analysis of covariance was performed to investigate the association of genotypes and this clinical index. Age, gender, exposure period, infection history, parasitemia levels and genetic ancestry, which are related to malaria infections and presents $p<0.15$ in exploratory analyses were included in the General Linear Model as covariates. General Linear Model analysis of covariance and Principal Component Analyses were performed using the SPSS18.0 statistical package for Windows®. Statistical significance was defined as a two-tailed P-value < 0.05.

RESULTS

In the present study, 147 symptomatic *P. vivax* malaria patients (65% males) aged between 12 and 88 years (35.2 ± 15.2 years) were included. Demographic and clinical features are summarized in Table 1. *IL4*, *IL4R*, *IL6* and *IL10* allele and genotype frequencies are shown in Table 2 whereas *IL10* derived haplotypes are described in Table 3. All Genotype distributions did not deviate significantly from Hardy-Weinberg equilibrium in the study population

The first component obtained in the Principal Component Analysis showed higher weights for fever, headache, chills, myalgia, arthralgia, back pain, abdominal pain, asthenia, dizziness and nausea, and explained 32,1% of symptom variability. This component was used as a clinical malaria severity index for association analyses.

In the General Linear Model of covariance (GLM) after adjustment for age, gender, exposure period, infection history, parasitemia levels, African and Native American genetic ancestry, *IL4* -590C>T and IL4R 1902A>G were not associated with the malaria clinical index ($p = 0.9$ and 0.09, respectively; Table 4).

Higher clinical index values were observed for *IL6* -174C carriers ($p = 0.0001$) and *IL10* -1082GG homozygotes ($p = 0.01$; with bonferroni correction). *IL10* -592A>C and -819C>T SNPs were not associated with the malaria clinical index, although non-significant -592CC and -819CC homozygotes showed higher values. Homozygotes for *IL10* haplotype

GCC (-1082/-819/-592) presented a higher clinical index when compared to ATA opposite haplotype heterozygotes and homozygotes (p = 0.03, and p = 0.02; with bonferroni correction respectively; Table 4).

DISCUSSION

Cytokines as interleukins are major players in immune response influencing and coordinating immune response based on the physiologic microenvironment induced by infections and inflammations. IL-4 is a major anti-inflammatory cytokine produced by CD4+ Th2 cells, basophils and mast cells. IL-4 regulates differentiation of Th2 effector cells, suppression of Th1 signaling, and promoting humoral immunity and Ig class switching (30,31). Higher IL-4 levels were observed in malaria intermediate and late stages (14). The IL4 gene is located in chromosome 5 (5q31-33), and a promoter -590C>T SNP was associated with *P. falciparum* malaria susceptibility and its cerebral form in Africans and Asians (13,15,16). However, in the present study this polymorphism was not associated with malaria symptom intensity in *P. vivax* infection. IL-4R is the IL-4 and IL-13 major receptor and when its function is blocked, Th2 immune response is impaired (12). In mice, IL4R expression in conjunction with CD8+ cells is required for memory response against malaria liver stage infection (32). Nonsynonymous polymorphisms found in the *IL4R* gene have been shown to modulate the IL-4 signal transduction pathway (33). Polymorphisms in this gene were recently associated with *P. vivax* malaria susceptibility in the study population (17). In the present study, the missense *IL4R* 1902A>G SNP (Q576R) was not associated with malaria symptoms intensity clinical index. Further studies in *IL4* and *IL4R* are warranted to determine the influence of these genes in malaria susceptibility and severity.

IL-6 is a pleiotropic cytokine with pro- and anti-inflammatory activities. In a classic signaling pathway, IL-6 binds to specific membrane IL-6 receptors (IL-6R) expressed in hepatocytes, macrophages, monocytes, neutrophils, B and T cells and exerts regenerative and anti inflammatory activities. Moreover, in a *trans-signaling* process, IL-6 utilizes the soluble IL-6R (sIL-6R) and binds to membrane GP130 protein exerting primarily pro inflammatory activities (34). The present study reports the association of -174C>G SNP in *IL6* 5' upstream region with *P. vivax* symptom severity. This promoter SNP was related with impaired *IL6* gene expression and IL-6 levels (35). The *IL6* -174C allele was

associated with reduced IL-6 circulating levels in healthy volunteers, but it has also been reported to be associated with higher IL-6 levels in neonates and adults with acute inflammations (35-37). Regarding malaria studies, circulating IL-6 levelswere higher in individuals with malaria (24,38,39). IL-6 levels were also associated with *P. falciparum* infection severity (23,40), hyperpyrexia and intensity of symptoms in *P. vivax* infection (24,29). The present study showed a higher clinical symptom index in *IL6* -174C allele carriers. Taken together this data suggest an important role for this IL6 in *P. vivax* malaria severity.

The immunoregulatory cytokine IL-10 has strong anti-inflammatory properties and can directly affect both the innate and the adaptive immune responses, preventing or limiting the development of an adequate immune response (41). Several studies investigated the role of IL10 in malaria infection with conflicting results. *IL10* -1082C>T SNP was not associated with IL-10 circulating levels or parasitemia in *P. vivax*-infected individuals (22), this allele was also associated with decreased IL-10 production in Mozambican young children with *P. falciparum* infections and malaria infections decreased risk (21). The GCC (-1082/-819/-592) haplotype was reported to be associated with increased IL-10 production and protection against severe malaria anemia in Kenyan children with *P. falciparum* malaria (20). In the present study the GCC (-1082/-819/-592) haplotype was associated with increased malaria severity that reflects the -1082G allele strong association with malaria severity. In animal models IL-10 cytokine has a well defined role controlling the inflammatory response and preventing severe clinical manifestations in malaria (42-45). In humans, high levels of this interleukin were associated with protection against severe anemia and asymptomatic *P. falciparum* malaria in an African population (20,46). Nevertheless, IL-10 high levels was also associated with cerebral malaria, severe anemia, and respiratory distress (39,47,48), and more recently, with *P. vivax* symptoms and paroxism intensity in an Amazonian population (29). The present work reports the influence *of IL10* polymorphisms in *P. vivax* malaria severity, the evidences so far indicate that this cytokine plays an important role in both *P. falciparum* and *P. vivax* infections.

IL-6 is extremely related to pro-inflammatory IL-1β and TNF-α signal transduction, but it also could play an important role in Th2 cell differentiation by inducing IL-4 expression (34,49). In inflammatory infection conditions IL-4 via IL4R could enhance IL-

10 production (50) that has an important regulatory role and can direct inhibit IL-1β, IL-6 and TNF-α cytokines or T cells proliferation and differentiation in Th1 or Th2 (18,51). This intricate network makes difficult to address the exactly biological function of each interleukin in malaria immune response *in vivo*. Although the present study shows *IL6 and IL10* polymorphisms influencing overall clinical symptom intensity in uncomplicated *P. vivax* malaria, the precise function of these polymorphisms in interleukin expression or immune system regulation is difficult to determine. Interleukin expression and circulating levels varies depending on distinct physiologic conditions and infections in humans with the same polymorphism allele in different population studies (20-22,35-37). Taking together with results presented herein, these data indicate that interleukin gene polymorphisms together with the physiologic microenvironment induced by different *Plasmodium* species are important factors for malaria severity and immune response in different populations.

Further functional studies that investigate how these genetic variants influence *P. vivax* specific symptoms and severe malaria in different populations will help to understand the role of cytokines in malaria pathology and immune response.

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TABLE 1 Study group characteristics

Characteristics	Malaria Patients
N	147
Age	35.2 (15.2)[a]
Gender (male %)	65.3
Exposure period (%)	
1 to 25 days	56.5
more than 25 days	25.2
Residant	18.3
Infections (%)	
First	69.4
Two	11.6
More than 2	19.0
Parasitemia (Parasites/μL)	8,606.1 (10,285.2)[a]
Genetic Ancestry	
African	0.245 (0.09)[a]
European	0.414 (0.11)[a]
Native American	0.341 (0.12)[a]

[a]Values expressed as mean ± SD

TABLE 2 *IL4*, *IL4R*, *IL6* and *IL10* genotypic and allelic frequencies

Gene		**dbSNP ID**	**N**	**Genotypes**			**Alleles**	
IL4	-590C>T	rs2243250	147	CC	CT	TT	C	T
				39 (26.6)	78 (53.0)	30 (20.4)	53.1	46.9
IL4R	1902A>G	rs1801275	147	AA	AG	GG	A	G
				47 (32.0)	82 (55.7)	18 (12.3)	59.9	40.1
IL6	-174C>G	rs1800795	147	GG	GC	CC	G	C
				102 (69.4)	43 (29.2)	2 (1.4)	84.0	16.0
IL10	-1082T>C	rs1800896	147	AA	AG	GG	A	G
				84 (57.1)	53 (36.1)	10 (6.8)	75.2	24.8
	-819C>T	rs1800871	147	TT	TC	CC	T	C
				18 (12.2)	76 (51.7)	53 (36.1)	38.1	61.9
	-592A>C	rs1800872	147	AA	AC	CC	A	C
				17 (11.6)	77 (52.4)	53 (36.0)	37.8	62.2

TABLE 3 *IL10* haplotype frequencies

Gene	Haplotype			Alelos	N	Frequency (%)
IL10	-1082T>C	-819C>T	-592A>C			
	A	C	C	1	110	37.4
	G	C	C	2	72	24.6
	A	T	C	3	1	0.3
	A	T	A	4	110	37.4
	G	T	A	5	1	0.3

TABLE 4 Clinical index according with *IL4*, *IL4R*, *IL6* and *IL10* genotypes and haplotypes

Gene	SNP	N	Clinical Index[a]	P
IL4	-590C>T			
	CC	39	-0.005 (-0.35:0.34)	
	CT	78	0.071 (-0.19:0.33)	0.9
	TT	30	0.007 (0.19:0.39)	
IL4R	1902A>G			
	GG	102	0.374 (-0.07:0.82)	0.09
	AA+AG	45	-0.032 (-0.27:0.21)	
IL6	-174C>G			
	GG	129	-0.136 (-0.38:0.10)	**0.001**
	GC+CC	18	0.392 (0.08:0.69)	
IL10	-1082T>C			
	AA	84	-0.088 (-0.35:0.18)	
	AG	53	0.063 (-0.22:0.35)	**0.01**[b]
	GG	10	0.832 (0.21:1.44)	
	-819C>T			
	TT	18	-0.199 (-0.67:0.28)	
	TC	76	-0.051 (-0.33:0.23)	0.19
	CC	53	0.207 (-0.91:0.50)	
	-592A>C			
	AA	17	-0.259 (-0.74:0.22)	
	AC	77	-0.038 (-0.31:0.24)	0.16
	CC	53	0.208 (-0.09:0.50)	
IL10 haplotype	-1082/-819/-592			
	ACC/ACC	23	0.013 (-0.41:0.43)	
	ATA/ATA	17	-0.259 (-0.73:0.21)	
	ATA/Others	76	-0.038 (-0.31:0.51)	**0.03**[c]
	GCC/Others	22	0.101 (-0.31:0.51)	
	GCC/GCC	9	0.955 (0.31:1.59)	

[a]Clinical index was adjusted in the model for age, gender, exposure period, infections history, parasitemia levels and african and native american genetic ancestry. Values are expressed as geometric mean and 95% confidence interval.

[b]*IL10* -1082T>C: AA vs AG p = 0.9, AA vs GG p = 0.01, AG vs GG 0.06, with bonferroni correction.

[c]*IL10* haplotypes: GCC/GCC vs GCC/Others p = 0.2, GCC/GCC vs ATA/Others p = 0.03, GCC/GCC vs ATA/ATA p = 0.02, GCC/GCC vs ACC/ACC p = 0.1, with bonferroni correction. All others pair wise analysis p > 0.05.

CAPÍTULO 6

THE EFFECT OF SINGLE NUCLEOTIDE POLYMORPHISMS IN CYTOCHROME P450 IN CHLOROQUINE /PRIMAQUINE *P. VIVAX* MALARIA TREATMENT IN BRAZIL

Manuscrito em preparação para ser submetido à revista Basic & Clinical Pharmacology &Toxicology

Title: **The effect of single nucleotide polymorphisms in cytochrome P450 in Chloroquine /Primaquine *P. vivax* malaria treatment in Brazil.**

Running title: **Cytochrome P450 SNP effect in *P. vivax* malaria treatment**

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Abstract:

Plasmodium vivax is a major cause of morbidity in the Amazon region. Cloroquine and primaquine combined regimen is the current therapy to eliminate *P. vivax* sexual and asexual forms in this region. The study of *CYP1A2*, *CYP2C8*, *CYP2C9*, *CYP3A4* and *CYP3A5* genetic polymorphisms influence in *P. vivax* malaria cloroquine/primaquine treatment was performed in a Brazilian population. A Generalized Estimating Equations analysis adjusted for covariates was performed to determine the genetic influence in parasitemia or gametocytemia clearence over treatment time. In these analyses, a significant effect of *CYP2C8* poor metabolizers (*2, *3, *4 carriers) on treatment was observed (P = 0.01). From baseline to the first day of treatment, *CYP2C8* wild-type individuals achieved greater reduction of gametocytes than poor metabolizers individuals (P = 0.007). *CYP2C9* gene and *CYP3A5* gene over time interactions for gametocytemia and parasitemia clearance rates were significant (P = 0.04 and P = 0.008), although after Bonferroni correction the statistical significance was lost. *CYP1A2* and *CYP3A4* genetic variants were not associated with cloroquine/primaquine response. Future studies must be performed to access *CYP2C8*, *CYP2C9* and *CYP3A5* gene polymorphisms influence in cloroquine/primaquine pharmacokinetics, adverse effects and treatment outcome.

Introduction

Individual variation in drug disposition and response make effective drug prescribing a clinical challenge. Differences in drug response make usual dosage regimes therapeutically effective in most patients, but some individuals do not experience any beneficial effect or suffer from drug toxicity. Genetic polymorphisms are major factors in phase I and phase II metabolizing enzymes influencing pharmacokinectics in drug response [1]. Tropical diseases usually need multiple drug therapies to control infections which make pharmacogenetic studies in such diseases still more complex.

Plasmadium vivax is the major cause of malaria disease outside Africa and it is an important morbidity and mortality factor in the Amazonian region [2]. The World Health Organization recommended as first choice treatment protocol for uncomplicated *P. vivax* malaria chloroquine (CQ) and primaquine (PQ) combined therapy [3]. CQ is a 4-aminoquinoline derivative of quinine, and has been the most widely used antimalarial drug since 1946 [4]. CQ is a potent schizontocide metabolized by cytochrome P450 (CYP) isozymes 2C8, 3A4, 3A5, and, to a lesser extent, 2D6 [5]. A quinine derivative, the 8-aminoquinoline PQ is an important gametocytocide and is the unique effective drug against *P. vivax* and *P. ovale* hypnozoites [6]. PQ is metabolized by CYP1A2 and CYP3A4 and forms carboxyprimaquine, an active metabolite [7,8]. This drug could lead to severe hemolytic anemia in subjects with glucose-6-phosphate dehydrogenase (G6PD) deficiency and this condition needs to be investigated before PQ prescription in some populations [6,9].

Interindividual variability in CQ and PQ concentrations and effect was reported in Africa and Asia which may affect treatment outcome in these populations [10-15]. *P. vivax* resistance to CQ and/or PQ, treatment non-compliance, medication suboptimum dose, patient health and/or nutritional status, drug-drug interactions are some factors which could lead to treatment failure [16]. However, genetic polymorphisms in CQ and PQ metabolizing enzymes that might influence drug availability and response to malaria therapeutic regimen were never investigated, therefore the present study aims to evaluate whether genetic polymorphisms in *G6PD*, *CYP1A2*, *CYP2C8*, *CP2C9*, *CP3A4*, *CYP3A5* influence *P. vivax* malaria treatment response.

Material and Methods

Study population. The study cohort consisted of 164 *P. vivax* malaria patients followed during malaria treatment period. All subjects were born in Pará state in the Brazilian Amazonian region. They were aged between 12 and 88 years (36.0 ± 15.6 years). Twenty nine patients (17.6%) use other medications in combination to CQ and PQ to treat malaria symptoms or pre-existing diseases. Sample collection and ancestry determination were previously described [17]. Patients were clinically examined and received the standard 1500 mg of CQ associated with 210 mg of PQ treatment [18]. The therapeutic regimen was administered as CQ 600 mg and PQ 30 mg in the first day, followed by CQ 450 mg and PQ 30 mg in the second and third days, and PQ 30 mg in the last four days. Treatment response was daily accompanied by clinical examinations. *P. vivax* asexual and sexual (gametocyte) forms density per μl of blood was daily estimated by counting the number of parasites per 100 fields and double-checked blindly by two expert microscopists. Patients were followed at the Evandro Chagas Institute for six months to identify relapse episodes. All subjects provided their written informed consent to participate in this study. The Ethics Committees of the Evandro Chagas Institute and Federal University of Pará approved the study protocol.

Genotyping. Genomic DNA from all patients was extracted from peripheral blood leukocytes subjects using proteinase K digestion and standard phenol-chloroform procedures [19]. The 13 SNPs in CYP450 and *G6PD* genes were determined by allelic discrimination with Taqman 5'-nuclease assays (Real Time PCR, Applied Biosystems, California, USA) according to the manufacturer's recommended protocol (Table 1).

Statistical analyses. Allele and genotype frequencies were estimated by gene counting, and haplotype frequencies and linkage disequilibrium were estimated with PHASE 2.1.1 [20]. Deviation from Hardy-Weinberg equilibrium was assessed by Qui-square tests with Bonferroni correction. The individual proportions of European, African and Amerindian genetic ancestry were estimated using the STRUCTURE software 2.3.3 [21,22]. Analyses of the effect of different genotypes on the efficacy of the treatment were performed using a Generalized Estimating Equations (GEE) to determine the genetic influence in parasitemia or gametocytemia clearence over time. GEE is a repeated measure analysis focused on average changes in response over time and the impact of covariates on these changes. This method models the mean response as a linear function of covariates of interest via a

transformation or link function and can be used in studies in which data are skewed or the distribution of data is difficult to verify due to small sample size [23]. GEE was performed considering a gaussian distribution with an identity link function and an independent correlation matrix structure in the SPSS18.0 statistical package for Windows®. Age, gender, co-medication, parasitemia baseline level, gametocytemia baseline level, and genetic ancestry entered in models as covariates based on conceptual analyses of the literature and/or by means of a statistical definition (association with the study factor and with the outcome at $P \leq 0.15$). Bonferroni correction for multiple comparisons was performed and corrected p values were presented. Cohen's *d* test was calculated to determine the effect sizes based on standardized differences between the means, that is, the difference between the means of the two conditions in terms of standard (z) scores [24]. Statistical significance was defined as a two-tailed P-value < 0.05.

Results

Based on *G6PD* 202G>A and 376A>G SNPs only three malaria patients showed Gd A^- deficiency and four women were 202A and 376G carriers. Allele and genotype frequencies for *G6PD* SNPs are presented in Table 2. The patients with G6PD deficiency did not present adverse reactions to CQ/PQ treatment, therefore *G6PD* genotypes were not considered as a confounder variable in this population study.

After 7 days of treatment all patients presented negative results for parasites and gametocytes in blood. Parasitemia levels were reduced to zero after five days of treatment and gametocytes was reduced to zero after four days of treatment. No patient abandoned treatment and adverse drug reactions were not reported. After treatment, 27 patients (16.5%) presented relapses and repeated the therapeutic regimen.

CYP1A2, *CYP2C8*, *CYP2C9*, *CYP3A4* and *CYP3A5* allele frequencies in the investigated sample are shown in Table 3. The genotype distribution did not deviate significantly from Hardy-Weinberg equilibrium. A functional approach was used to group genotypes. Therefore, poor metabolizer carriers were compared to subjects with normal metabolism to explore the effect of these genes on outcomes. After adjustment for age, gender, co-medication, parasitemia baseline level, gametocytemia baseline level, and genetic ancestry in the GEE analysis, only *CYP2C8*, were associated with gametocytemia clearance rates.

CYP2C8 reduced activity variants (*2, *3, *4) carriers were considered as poor metabolizers. Demographic and clinical characteristics of the patients according to metabolism status are shown in Table 4. Figure 1 shows the trajectory of gametocyte elimination based on findings from the GEE model, including treatment over time and the presence of poor metabolizer genotypes as main effects, age, gender, co-medication, gametocytemia baseline level, and genetic ancestry as covariates (conceptual confounders), and significant interactions between these factors during treatment. A significant effect of *CYP2C8* poor metabolizers ($P_{Bonferroni}$ = 0.01) on treatment was observed and a significant interaction effect between poor metabolism and treatment over time (P = 0.014) was also observed although after Bonferroni correction it was no longer significant. From baseline to the first day of treatment, homozygous individuals for wild-type *CYP2C8* achieved greater reduction (P = 0.007) of gametocytes than individuals without this genotype (Figure 2). The effect size of the *CYP2C8* polymorphism represent moderate clinical effects considering Cohen's suggestion that an effect size of 0.44 indicates a moderate effect [25].

CYP2C9 gene were associated with gametocytemia clearance rates (p = 0.04), but this association after Bonferroni correction was no longer significant (Table 5). No main effect was observed for *CYP3A5* But an interaction between gene over time on parasitemia elimination rate during treatment was disclosed (P = 0.008) (Table 5). *CYP3A5*3* and **6* carriers showed a lower rate of parasite elimination rate during treatment compared with wild type carriers. After Bonferrorni correction only a trend for these associations were observed, and were not further explored.

Discussion

Metabolism plays an important role in drug disposition with pharmacological and toxicological implications in the use of therapeutic drugs. CYPs are expressed mostly in the liver representing the most important phase I drug-metabolizing enzymes that oxidize several endogenous substances and xenobiotics, as most medications [26].

The human *CYP2C8* and *CYP2C9* genes are mapped to chromosome 10q24 and exhibit similar substrate specificity but with distinct metabolizing rates [27,28]. CYP2C8 is mainly expressed in the liver and metabolizes near 5% of drugs cleared by phase I reactions, while CYP2C9, an abundant enzyme expressed in the liver, metabolizes

approximately 15% of clinical drugs [29,30]. In the present study, *CYP2C8* reduced activity alleles carriers showed lower rates of gametocyte elimination as compared with homozygous wild type allele **1A* carriers. *CYP2C8*4* is a missense mutation which promotes a lower enzyme activity in vitro than the wild type allele **1A* [31]. *CYP2C8*2* and *CYP2C8*3* also presents a markedly decrease activity in vitro [32]. *CYP2C8*2* and *CYP2C8*3* were associated with impaired metabolism of the antimalarial amodiaquine in Africa [33]. In that study *CYP2C8*4* has not been identified. The lower rate of gametocyte elimination by poor metabolizers observed herein should be better evaluated in further studies, because it could indicate a slow response to treatment and could be associated with more adverse effects during treatment or with worse response. *CYP2C9* reduced activity allele carriers showed a lower gametocytemia clearance rate during treatment period, although not significant after Bonferroni correction. CYP2C9 was not related to CQ or PQ metabolism, however, linkage disequilibrium between *CYP2C9* and *CYP2C8* alleles were reported [34]. In the admixed Brazilian population *CYP2C8* and *CYP2C9* genes constitute a haplotype block [35]. Linkage disequilibrium or an overlap of these enzyme functions are a possible explanation for the trend observed in CQ/PQ treatment outcome.

The present study also reported a trend for a gene over time interaction between lower parasite elimination rates during malaria treatment and *CYP3A5* splicing defect alleles (*CYP3A5*3* and **6*) carriers.

The present study demonstrated that *CYP2C8*, *CYP2C9*, *CYP3A5* genetic variants potentially influence in CQ/PQ malaria treatment. CQ has a major effect as schizontocides in erythrocytes, but this medication also presents some effect in *P. vivax* gametocytes. PQ has major effect as gametocytocide and as hypnozoitocide in liver and is metabolized mainly by CYP1A2 and CYP3A4.

CYP2C8 alleles were associated with gametocytemia clearance in CQ/PQ associated regimen, however CQ is less effective in gametocyte elimination than PQ and the genetic effect reported probably represent the synergistic effect of both drugs. The study design does not allow the investigation of the genetic influence on malaria relapses. A long term study is necessary to investigate pharmacogene polymorphisms on *P. vivax* malaria relapses.

Anti malarial drugs were usually administered in combination therapies making difficult pharmacogenetics and pharmacokinetics data interpretation. The present study

was performed with patients in normal treatment conditions and differences in age, gender, genetic ancestry and use of other drug together with malaria treatment were taken into account in the analyses. Multiple comparison correction tests and effect size estimates were also performed to address reliable results. Nevertheless, the study has some limitations: (1) it was not possible to infer or control for the interindividual immune response variability in malaria patients, that contributes to malaria treatment response; (2) it was not possible to determine if patients were *P. vivax*-infected with CQ or PQ resistant strains, however the patients did not present relapses before 28 days which is considered a CQ resistance *in vivo* test [36], (3) CQ and PQ plasma concentrations were not assessed and CYP genetic variance influence in its pharmacokinetics could not be directly correlated. Besides these limitations, the present results and the effect size reported reinforce the potential of pharmacogenomics to *P. vivax* malaria treatment.

Future studies with larger sample sizes are clearly warranted to access if *CYP2C8*, *CYP2C9* and *CYP3A5* gene polymorphisms to confirm the influence in CQ/PQ in treatment outcome.

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Table 1 List of SNPs genotyped in the present study

Gene	SNP	dbSNP ID	Assay ID
CYP1A2	-360G>A	rs2069514	C__15859191_30
	-163C>A	rs762551	C__8881221_40
CYP2C8	805A>T	rs11572103	C__30634034_10
	792C>G	rs1058930	C__25761568_20
	416G>A	rs11572080	C__25625794_10
CYP2C9	3608C>T	rs1799853	C__25625805_10
	1003C>T	rs28371685	C__30634132_70
	42614A>C	rs1057910	C__27104892_10
	1080C>G	rs28371686	C__27859817_40
CYP3A4	-392A>G	rs2740574	*
CYP3A5	14690G>A	rs10264272	C__30203950_10
	6986A>G	rs776746	C__26201809_30
G6PD	202G>A	rs1050828	C__2228686_20
	376A>G	rs1050829	C__2228694_20

*Custom assay

Table 2. *G6PD* allele and genotype frequencies

SNP	Alleles			Genotypes	
		Male	Female		
202G>A	G	105 (98.1%)	89 (93.0%)	GG	43 (89.6%)
	A	2 (1.9)	7 (7.0%)	GA	3 (6.3%)
				AA	2 (4.2%)
376A>G	A	94 (88.7%)	86 (91.5%)	AA	40 (85.1%)
	G	12 (11.3%)	8 (8.5%)	AG	6 (12.%)
				GG	1 (2.1%)

*202A + 376G determines A^- phenotype

Ttable 3. CYP450 allelic frequencies.

Gene	Alleles	N	Frequency (%)
CYP1A2	*1A	122	37.2
	*1C	127	38.8
	*1F	79	24.0
CYP2C8	*1A	283	86.2
	*2	19	5.8
	*3	21	6.4
	*4	5	1.5
CYP2C9	*1A	291	88,6
	*2	23	7.2
	*3	9	2.8
	*11	5	1.5
CYP3A4	*1A	272	83.0
	*1B	56	17.0
CYP3A5	*1A	80	24.5
	*3	235	71.8
	*6	12	3.7

Table 4. CYP2C8 group phenotypes main characteristics

Characteristics	Normal Metabolizer	Poor metabolizer	*P*
N	118	45	
Age	36.0 (15.6)	35.0 (15.1)	0.6
Gender (male %)	68.1	71.1	0.4
Baseline Parasitemia (Parasites/μL)	8,375.35 (8,692.0)	9,222.1 (1,3370.4)	0.6
Baseline Gametocytemia (Gametocytes/ μL)	126.4 (465.0)	73.33 (136.3)	0.6
Genetic Ancestry			
African	0.239 (0.96)	0.254 (0.95)	0.2
European	0.415 (0.10)	0.416 (0.13)	0.6
Native American	0.345 (0.12)	0.329 (0.13)	0.5

Values for age parasitemia, gametocytemia and genetic ancestry are expressed as mean (SD)

P values: Student t test for age; Fisher exact test for gender; Mann-Whitney test for parasitemia and gametocytemia baseline and genetic ancestry.

Table 5. Parasitemia and gametocytemia reduction association with *CYP* genes and gene interaction with time

Gene	Parasitemia					Gametocytemia				
	P		$P_{Bonferroni}$		d*	*P*		$P_{Bonferroni}$		d*
	Gene	Gene*Time	Gene	Gene*Time		Gene	Gene*Time	Gene	Gene*Time	
CYP1A2	0.7	0.1	ns	ns		0.2	0.3	ns	ns	
CYP2C9	0.8	0.1	ns	ns		0.043	0.1	0.43	ns	0.24
CYP3A4	0.8	0.2	ns	ns		0.7	0.8	ns	ns	
CYP3A5	0.5	0.008	ns	0.08	0.53	0.5	0.6	ns	ns	

*Effect size Cohen's d test

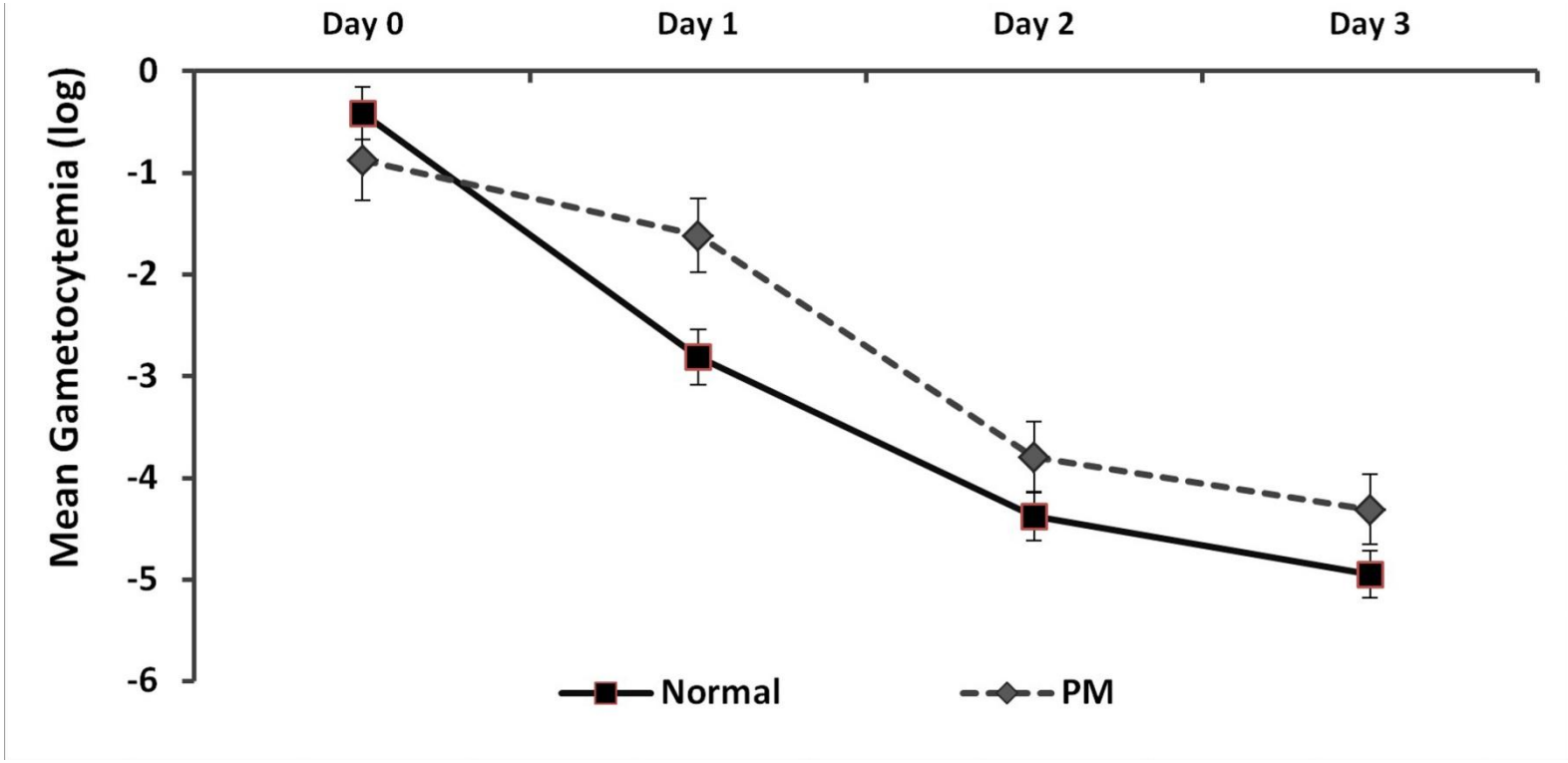

Figure 1. Mean gametocytemia level reduction during choloroquine/primaquine regimen according to *CYP2C8* phenotypes. Generalized Estimating Equations method with age, gender, co-medication, gametocytemia baseline level and genetic ancestry as co-variates; $P_{Bonferroni} = 0.01$ and d = 0.44.

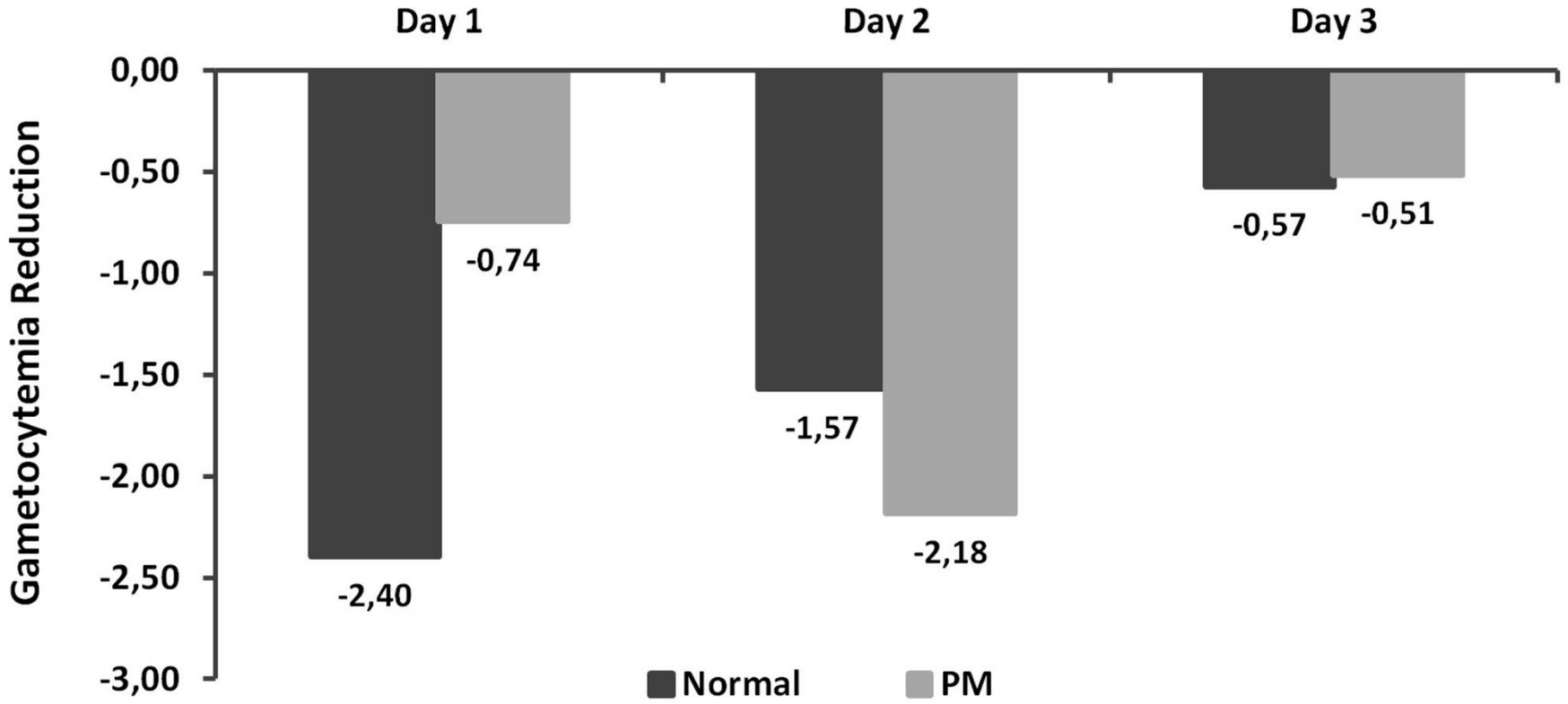

Figure 2. Effect in mean gemetocytemia reduction from baseline during choloroquine/primaquine regimen comparing *CYP2C8* phenotypes. Generalized Estimating Equations method. Day 1 p = 0.007; day 2 p = 0.15; and day 3 p = 0.10.

CAPÍTULO 7

TRANSPORTER GENE POLYMORPHISMS INFLUENCES CHLOROQUINE AND PRIMAQUINE IN *P. VIVAX* MALARIA TREATMENT

Manuscrito em preparação para ser submetido à revista Pharmacogenetics and Genomics

Title: ***ABCB1*, *SLCO1B1* and *SLCO2B1* transporter gene polymorphisms influences chloroquine and primaquine in *P. vivax* malaria treatment**

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Abstract

Objective To investigate if genetic polymorphisms in efflux and uptake transporters *ABCB1, ABCC2, ABCC4, ABCG2, SLCO1A2, SLCO1B1, SLCO1B3* and *SLCO2B1* influence *P. vivax* malaria treatment response with chloroquine and primaquine in a Brazilian population.

Method *P. vivax* malaria patients (n = 164) who were born in the Brazilian Amazonian region received standard chloroquine/primaquine treatment. Patients were followed up during treatment, and parasitemia and gametocytemia were daily estimated. All patients were genotyped for 19 polymorphisms in transporters genes. A Generalized Estimating Equations analysis adjusted for covariates was performed to determine the genetic influence in parasitemia or gametocytemia clearence over treatment time.

Results The interaction among *ABCB* and *SLCO2B1* genotype treatment over time were associated with the rate of parasitemia. *ABCB1* homozygous individuals for the wild-type CGC/CGC haplotype have higher parasitemia clearance rates than TnonGT carriers (P_{bonf} = 0.03). *SLCO2B1* AA carriers showed a higher parasitemia elimination rates than AG and GG genotypes (P_{bonf} < 0.001). *SLCO1B1* gene treatment over time interaction was associated with gametocytemia clearance rates in malaria treatment (p_{bonf} = 0.04). *SLCO1B1*14* carriers showed a lower gametocytemia elimination rates compared with other allele carriers. In GEE analysis *ABCC2, ABCC4, ABCG2, SLCO1A2* and *SLCO1B3* gene main effect or gene over time interaction were not associated with malaria treatment response

Conclusion The present work presents the first pharmacogenetic report of an association between chloroquine/primaquine responses with transporter genes. Therefore, the present results should be replicated in larger and independent samples.

Keywords: Malaria, *P. vivax*, ABCB1, SLCO1B1, SLCO2B1, Chloroquine, Primaquine, Pharmacogenomics

Introduction

Plasmodium vivax is the major cause of malaria outside Africa and represents a real challenge for malaria eradication in Asia and America continents. This parasite has the intrinsic characteristic to develop dormant hypnozoite forms in the liver that cause subsequent infections in the blood [1].

The World Health Organization recommends chloroquine (CQ) and primaquine (PQ) combined therapy as first choice treatment protocol for uncomplicated *P. vivax* malaria [2]. The quinine derivatives CQ and PQ combined therapy targets are asexual schizonts in blood and tissues, sexual gametocytes in blood and also hypnozoites in the liver [3-5].

Interindividual variability in CQ and PQ concentrations were reported in different populations [6-11]. Although antimalarial treatment and prophylaxis is primarily through chemotherapy, little is known about the relation between the dose of antimalarial drugs, exposure to metabolites, and therapeutic response. The metabolic pathways of antimalarial drugs have been established only recently, while the role of efflux and uptake transport remains largely unexplored [12].

Transporters are integral membrane proteins that mediate the translocation of chemicals into and out of cells using active and passive mechanisms [13]. ATP-binding cassette (ABC) and solute carrier transporters families are formed by influx and efflux transporters expressed on membranes of polarized cells and have been shown to significantly affect concentrations of drugs in plasma and peripheral tissues, thus affecting drug efficacy and toxicity [14,15]. Membrane transporters as multidrug resistance protein 1 (MDR1), multi drug proteins (MRP) and breast cancer resistance protein (BCRP) are members of the ATP-binding cassette family and utilize ATP to move substrates across membranes. Instead, the organic anion transporting proteins (OATPs) moves substrates against a concentration gradient without ATP expend, and together with ABC transporters are responsible for transport and availability of several endogenous and exogenous compounds [14]. CQ seems to be an ATP-binding transporters inhibitor; however some evidences indicate that this medication is a potential substrate for these transporters. The MRP transport system is responsible for CQ cellular direct efflux in multidrug-resistant tumor cells [16,17], and this drug also could act as substrate or inhibitor of human MDR1 transporter [18,19]. PQ also inhibits MDR1 and MRP1 drug transport without being a

substrate [19], but the transporter effect in PQ pharmacokinetic properties was not reported.

Genetic polymorphisms in efflux and uptake transporters and CQ and PQ were not been assessed, therefore the present study aims to investigate if *ABCB1, ABCC2, ABCC4, ABCG2, SLCO1A2, SLCO1B1, SLCO1B3* and *SLCO2B1* variability influence *P. vivax* malaria treatment response in a Brazilian population.

Material and Methods

Study population

The study population was composed by 164 *P. vivax* malaria patients who were born in Pará state in the Brazilian Amazonian region. The sample and collection procedures were previously described [20].

Patients were clinically examined and received the standard treatment 1500 mg of CQ associated with 210 mg of PQ in a week (first day CQ 600 mg and PQ; second and third days CQ 450 mg and PQ 30 mg; last days PQ 30mg) [21]. Patient treatment response was daily accompanied by clinical examinations and parasitemia and gametocytemia were daily estimated. All subjects provided their written informed consent to participate in this study. The Ethics Committees of the Evandro Chagas Institute and Federal University of Pará approved the study protocol.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using proteinase K digestion and standard phenol-chloroform procedures [22]. Single-nucleotide polymorphisms (SNPs) in *ABCB1, ABCC2, ABCC4, ABCG2, SLCO1A2, SLCO1B1, SLCO1B3* and *SLCO2B1* genes were determined by allelic discrimination with Taqman 5'-nuclease assays (Real Time PCR, Applied Biosystems, California, USA) according to the manufacturer's recommended protocol (Supplemental Table 1).

Statistical analyses

Allele and genotype frequencies were estimated by gene counting. Deviation from Hardy-Weinberg equilibrium was assessed by Qui-square tests with Bonferroni correction. Haplotype frequencies and linkage disequilibrium were estimated with PHASE 2.1.1 [23]. The individual proportions of European, African and Amerindian genetic ancestry were estimated as described [24].

A Generalized Estimating Equations (GEE) is a repeated measure analysis focused on average changes in response over time and the impact of covariates on these changes. This method models the mean response as a linear function of covariates of interest via a transformation or link function and can be used in studies in which data are skewed or the distribution of data is difficult to verify due to a small sample size [25]. This analysis was performed to determine the genetic influence in parasitemia or gametocytemia clearence by treatment over time considering a gaussian distribution with an identity link function and an independent correlation matrix structure. Age, gender, co-medication, parasitemia baseline level, gametocytemia baseline level, and genetic ancestry entered in models as covariates based on conceptual analyses of the literature and/or by means of a statistical definition (association with the study factor and with the outcome at $P \leq 0.15$). GEE analysis was performed with the SPSS18.0 statistical package for Windows®. Bonferroni correction for multiple comparisons was performed and corrected p values were presented. Cohen's *d* test was calculated to determine the effect sizes based on standardized differences between the means, that is, the difference between the means of the two conditions in terms of standard (z) scores [26]. Statistical significance was defined as a two-tailed P-value < 0.05.

Results

Major demographic and clinical features from the study population are summarized in Table 1. Malaria patients were aged between 12 and 88 years (36.0 ± 15.6 years) and 29 patients (17.6%) used other medications in combination with CQ/PQ treatment to manage malaria symptoms or pre-existing diseases. No patient abandoned treatment and adverse drug reactions were not reported. After complete treatment all patients presented negative results for parasites and gametocytes in blood.

ABCB1, ABCC2, ABCC4, ABCG2, SLCO1A2, SLCO1B1, SLCO1B3 and *SLCO2B1* allele and genotype frequencies are shown in Supplemental Table 2. Haplotype frequencies are shown in Supplemental Table 3. Genotype distributions did not deviate significantly from Hardy-Weinberg equilibrium in the study population.

After adjustment for age, gender, co-medication, parasitemia baseline level, and genetic ancestry, *ABCB1,* and *SLCO2B1* genes were associated with the rate of parasitemia over treatment time. No main gene effect was observed for both genes (Table 2).

Figure 1 shows the trajectory of parasite elimination based on findings from the GEE model, including treatment over time and the presence of ABCB1 more common haplotypes as main effects, age, gender, co-medication, parasitemia baseline level, and genetic ancestry as covariates, and significant interactions between these factors during treatment. A significant interaction effect between CGC/CGC homozygotes and treatment over time was observed (p_{bonf} = 0.03). Homozygous individuals for the wild-type haplotype have higher parasitemia clearance rates than TnonGT carriers. From baseline to the second day of treatment, homozygous individuals for CGC haplotype achieved greater reduction of parasites (P = 0.003) than individuals without this genotype (Figure 2). The effect size of this interaction is moderate considering Cohen's suggestion that an effect size of 0.47 indicates a moderate effect [27].

Figure 3 shows the model including treatment over time, the presence of *SLCO2B1* genotypes, and their interaction. Although no effect of genotype was detected (p=0.1), there was a significant interaction effect between genotypes and treatment over time for parasitemia elimination rates during treatment (p_{bonf} < 0.001). *SLCO2B1* AA carriers showed a higher parasitemia elimination rates than AG and GG genotypes (Figure 3). The effect size of this interaction is big considering Cohen's suggestion that an effect size of 0.89 indicates a large effect [27]. Figure 4 shows that the greatest effect of treatment occurred from baseline to the second day. AA homozygotes had a faster rate of parasite elimination than their counterparts with other genotypes.

SLCO1B1 was the only transporter gene associated with gametocytemia clearance rates in malaria treatment. Although no effect of genotype was detected (p = 0.09), there was a significant interaction effect between genotype and treatment over time (p_{bonf} = 0.04). *SLCO1B1*14* carriers showed a lower gametocytemia elimination rates compared with other allele carriers (Figure 5). Following effect size Cohen's interpretation scale *SLCO1B1* models showed big effect sizes (d = 1.20). Allele *1A carriers have a faster rate of gametocyte elimination, at day 2, carriers of this allele had no more gametocytes in their blood (Figure 6)

In GEE analysis adjusted for all covariates, *ABCC2, ABCC4, ABCG2, SLCO1A2* and *SLCO1B3* gene or gene over time interaction were not associated with malaria treatment response.

Discussion

The main finding of the present study was that both ABC and solute carrier transporters influence *P. vivax* malaria treatment in a Brazilian Amazonian population. Malaria control has traditionally relied upon two approaches: killing the mosquito vector and employing effective chemotherapy and chemoprophylaxis. The potential role of drug transporters in antimalarial treatment became clear after the observation that the malaria parasite expresses transporter genes in its digestive vacuole as defense mechanism against, for example, chloroquine. It is likely that there is a large overlap in substrate specificity between drug transporters in *Plasmodium* spp and humans, so genetic variants of drug transporters in humans might foster the development of drug resistance by, for example, lowering drug concentrations in red blood cells [12]. CQ and PQ have a synergistically effect as schizontocide, gametocide and hipnozontocide, and could be either substrates or inhibitors for these transporters. Nevertheless pharmacogenomic studies with drug transporter polymorphism for the most common drugs employed for uncomplicated malaria are scarce.

ABC transporter superfamily comprises membrane proteins that translocate a variety of substrates across cellular membranes acting as efflux proteins [28]. MDR1, also known as P-glycoprotein (P-gp) has several polymorphisms [29], among them SNPs 1236C>T, 2677G>T/A and 3435C>T. These SNPs located in exons 12, 21 and 26, respectively, are in strong linkage disequilibrium and have been the most commonly investigated [30,31]. MDR1 is responsible for the active efflux of many drugs, by biliary and renal excretion [32]. In the present study parasitemia and gametocytemia clearance during CQ/PQ regimen was associated with *ABCB1* gene haplotypes. *ABCB1* TnonGT carriers showed a slower parasitemia clearance rate when compared to wild type haplotype CGC homozygotes. Chloroquine seems to be a broad inhibitor of ABC transporters and a potential substrate of some ABCs. We have shown that *ABCB1* is *a* promising target, particularly in regard to chloroquine-induced adverse drug effects, which have been related to drug concentrations and pharmacogenetic vulnerability.

Solute carriers OATPs are plasma membrane proteins that mediate the active cellular influx of a variety of amphipathic compounds [33]. These proteins were expressed in apical and basolateral membranes of polarized cells in tissues such as liver and kidneys, as well as in the intestinal wall and the blood–brain barrier, and may affect pharmacokinetics

and effects of their substrates [34]. The nonsynonymous 935G>A SNP in *SLCO2B1* was associated with parasitemia elimination rates in malaria treatment in the study population. Homozygous patients for the 935A allele showed a significant higher parasitemia clearance rate when compared to GG homozygotes. The heterozygous 935A/G showed an intermediate rate. OATP2B1 also is expressed in the sinusoidal membrane of hepatocytes in the luminal membrane of small intestinal enterocytes, suggesting that it participate in drugs uptake from blood and absorption [35,36]. The precise influence in CQ and PQ pharmacokinetics and availability still needs to be determinated

OATP1B1 and OATP1B3 are mainly expressed in the sinusoidal membrane of human hepatocytes, however the OATP1B1 is expressed diffusely in the liver lobulus while OATP1B3 is expressed more perivenously [37]. These proteins mediates uptake of its substrates from blood into the liver and presents an important substrate overlapping [38]. OATP1B1 is encoded by *SLCO1B1* and SNPs in these genes were well characterized and associated with statins pharmacogenetics [39-42]. In the present study, the *SLCO1B1*14* allele carriers have a lower gametocytemia elimination rate in malaria treatment. This allele is formed by 388G and 463A SNP variants which was associated with reduced transport activity in vitro [43] and also with fluvastatin treatment response [40]. OATP1B3 is a major uptake transporter for docetaxel, a chemotherapy medication, and genetic polymorphisms in *SLCO1B3* were associated with this drug disposition in Chinese patients [44]. Genetic variants studied in the present work do not influence CQ/PQ response.

The observational-naturalistic design of our study, moderate sample size and the absence of CQ/PQ plasma level information in our patients are limitations of the present study. However, this design might be valuable to better appreciate the role of genetic factors in routine clinical practice beyond the realm of controlled clinical trials, but some caveats of this kind of studies should be considered. First, we had no internal control to correct for any effect of time (*eg*, regression to the mean) or expectancy bias, because we did not have a placebo arm in this trial. Second, we did not control for parasite resistance. Nevertheless, CQ-resistant *P. vivax* has been estimated to varied from 4.4% to 10% in the Amazonian region [45] and all patients presented negative results after treatment. The role of transporter gene variability is associated with the rate of clearance and not with efficacy *per se*. Although it is not possible to exclude that the effects we observed were due to lack

of adherence to treatment there is no reason to expect a preferential compliance to CQ/PQ treatment according to transporter gene genotypes.

This study design also did not allow to evaluate PQ effect in liver hypnozoites, or to evaluate the effect of transporter genes in relapses occurrence. Despite these considerations, the clinical relevance of this report resides in the fact that it is the first pharmacogenetic report of an association between CQ/PQ responses with transporter genes. Therefore, the present results should be replicated in larger and independent samples.

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Conflicts of interest

The authors declare that they have no conflict of interests

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Table 1 Study group main characteristics

Characteristics	Malaria Patients
N	164
Age	36.0 (15.6)
Gender (male %)	68.5
Baseline Parasitemia (Parasites/μL)	8,554.35 (10,146.20)
Baseline Gametocytemia (Gametocytes/ μL)	110.93 (401.879)
Genetic Ancestry	
African	0.243 (0.09)
European	0.415 (0.11)
Native American	0.340 (0.12)
Co-medication (%)	
Antiemetic	4.2
Antipyretic/Analgesic	12.2
Antacid	2.4
Antibiotic	1.2
Anthelmintic	0.6
Anticonvulsant	0.6
ACE inhibitor	0.6

Values for age, parasitemia, gametocitemia and genetic ancestry are expressed as mean (SD)

Table 2. Mean parasitemia over treatment time according to *ABCB1* and *SLCO2B1* genes.

Gene	Treatment	Parasitemia (parasites/µL)			*P*		$P_{Bonferroni}$		*d**
					Gene	Gene*time	Gene	Gene*time	
ABCB1		CGC/CGC (n=43)	TnonGT (n=83)						
	Day 1	329,9 (172,8;629,7)	248.6 (144.4;428.0)						
	Day 2	0.33 (0.09;1.21)	0.86 (0.33;2.26)		0.2	0.002	**NS**	**0.03**	0.48
	Day 3	0.01 (0.01;0.01)	0.03 (0.01;0.05)						
	Day 4	0.01 (0.01;0.02)	0.01 (0.0;0.01)						
SLCO2B1		AA	AG	GG					
	Day 1	337.8 (178.0;641.1)	352.4 (236.6;524.8)	211.1 (85.5;521.1)					
	Day 2	0.11 (0.02;0.50)	0.89 (0.34;2.37)	3.55 (1.07;11.85)	0.1	<0.001	NS	**<0.001**	0.89
	Day 3	0.01 (0.01;0.02)	0.02 (0.01;0.04)	0.02 (0.01;0.03)					
	Day 4	0.01 (0.0;0.02)	0.01 (0.0;0.01)	0.0 (0.0;0.01)					

Mean parasitemia adjusted for age, gender, co-medication, gametocytemia baseline level

Parasitemia is expressed as geometric mean (95% CI)

*Effect size Cohen's d test

NS = no significance

Supplemental Table 1 List of SNPs genotyped in the present study

Gene	SNP	Effect	dbSNP ID	Assay ID
ABCB1	3435C>T	Synonymous	rs1045642	C___7586657_20
	2677G>A/T	S893T	rs2032582	#
	1236C>T	Synonymous	rs1128503	C___7586662_10
ABCC2	-24C>T	5' UTR	rs717620	C___2814642_10
	1249G>A	V417I	rs2273697	C__22272980_20
	3563T>A	V1188E	rs17222723	C__25591743_30
	3972C>T	Synonymous	rs3740066	C__11214910_20
	4544G>A	C1515Y	rs8187710	C__22272567_30
ABCC4	912G>T	K304N	rs2274407	C__16181780_20
ABCG2	421C>A	Q141K	rs2231142	C__15854163_70
	34G>A	V12M	rs2231137	*
SLCO1A2	516A>C	E172D	rs11568563	C__25605897_10
	38T>C	I13T	rs10841795	C__25605906_20
SLCO1B1	388A>G	N130D	rs2306283	C___1901697_20
	463C>A	P155T	rs11045819	*
	521T>C	V174A	rs4149056	C__30633906_10
SLCO1B3	334T>G	S112A	rs4149117	C__25639181_40
	699G>A	M233I	rs7311358	C__25765587_40
SLCO2B1	935G>A	R290Q	rs12422149	C___3101331_10

Assay C_11711720D_40 for 2677G>A alleles and assay C_11711720C_30 for 2677G>T alleles.

*Custom assay

Supplemental Table 2. Transporter genes allelic and genotypic frequencies

Gene	SNP	dbSNP ID	N	Genotypes			Alleles	
ABCB1	3435C>T	rs1045642		CC	CT	TT	C	T
			164	55 (33.5)	72 (43.9)	37 (22.6)	182 (56.0)	146 (44.0)
	2677G>A/T	rs2032582		GG	G/NonG	NonG	G	NonG
			164	70 (42.7)	74 (45.2)	20 (12.1)	214 (65.2)	114 (34.8)
	1236C>T	rs1128503		CC	CT	TT	C	T
			164	60 (36.6)	74 (45.2)	30 (18.2)	194 (59.1)	134 (40.9)
ABCC2	-24C>T	rs717620		GG	GA	AA	G	A
			164	126 (76.8)	36 (22.0)	2 (1.2)	288 (87.8)	40 (12.2)
	1249G>A	rs2273697		GG	GA	AA	G	A
			164	120 (73.2)	41 (25.0)	3 (1.8)	281 (85.7)	47 (14.3)
	3563T>A	rs17222723		TT	TA	AA	T	A
			164	150 (91.4)	13 (8.0)	1 (0.6)	313 (95.5)	15 (4.5)
	3972C>T	rs3740066		GG	GA	AA	G	A
			164	72 (43.9)	79 (48.1)	13 (8.0)	223 (68.0)	105 (32.0)
	4544G>A	rs8187710		AA	AG	GG	A	G
			164	2 (1.2)	20 (12.2)	142 (86.6)	24 (7.3)	304 (92.6)
ABCC4	912G>T	rs2274407		CC	CA	AA	C	A
			164	131 (79.3)	33 (20.1)	1 (0.6)	293 (89.4)	35 (10.6)
ABCG2	421C>A	rs2231142		CC	CA	AA	C	A

			164	125 (76.3)	33 (20.1)	6 (3.6)	283 (86.3)	45 (13.7)
	34G>A	rs2231137		AA	AG	GG	A	G
			164	6 (3.6)	51 (31.1)	107 (65.3)	63 (19.2)	265 (80.2)
SLCO1A2	516A>C	rs11568563		AA	AC	CC	A	C
			164	154 (93.9)	10 (6.1)	0	318 (97.0)	10 (3.0)
	38T>C	rs10841795		AA	AG	GG	A	G
			164	146 (89.0)	17 (10.4)	1 (0.6)	309 (94.8)	19 (5.8)
SLCO1B1	388A>G	rs2306283		AA	AG	GG	A	G
			164	32 (19.5)	90 (54.9)	42 (25.6)	154 (46.9)	174 (53.1)
	463C>A	rs11045819		CC	CA	AA	C	A
			164	141 (86.0)	22 (13.4)	1 (0.6)	304 (92.7)	24 (7.3)
	521T>C	rs4149056		TT	TC	CC	T	C
			164	5 (3.0)	49 (29.9)	110 (67.7)	59 (17.9)	269 (82.1)
SLCO1B3	334T>G	rs4149117		TT	TG	GG	T	G
			164	10 (6.2)	65 (39.5)	89 (54.3)	85 (25.9)	243 (74.1)
	699G>A	rs7311358		GG	GA	AA	G	A
			164	10 (6.2)	65 (39.5)	89 (54.3)	85 (25.9)	243 (74.1)
SLCO2B1	935G>A	rs12422149		GG	GA	AA	G	A
			164	53 (32.3)	84 (51.2)	27 (16.6)	190 (57.9)	138 (42.1)

Supplemental Table 3. Transporters gene haplotype frequencies

Gene	Haplotypes					N	Frequency (%)
ABCB1	3435C>T	2677G>A/T	1236C>T				
	T	G	T			15	4.6
	T	G	C			25	7.7
	T	NonG	T			101	30.7
	T	NonG	C			5	1.5
	C	G	T			15	4.6
	C	G	C			160	48.8
	C	NonG	T			3	0.9
	C	NonG	C			4	1.2
ABCC2	-24C>T	1249G>A	3563T>A	3972C>T	4544G>A		
	A	G	T	A	G	39	11.9
	A	G	T	G	G	1	.3
	G	G	T	A	G	63	19.2
	G	G	T	G	G	154	47.0
	G	G	T	G	A	9	2.7
	G	G	A	G	A	15	4.6
	G	A	T	A	G	2	.6
	G	A	T	G	G	45	13.7

ABCG2	421C>A	34G>A			
	C	G		221	67.4
	C	A		63	19.2
	A	G		44	13.4
SLCO1A2	516A>C	38T>C			
	C	A		10	3.1
	A	A		298	91.0
	A	G		19	5.9
SLCO1B1	388A>G	463C>A	521T>C		
*1a	A	C	T	150	45.7
*5	A	C	C	4	1.2
*1B	G	C	T	95	29.0
*15	G	C	C	55	16.8
*14	G	A	T	24	7.3
SLCO1B3	334T>G	699G>A			
	G	A		243	74.1
	T	G		85	25.9

Figure 1. Mean parasitemia level reduction during choloroquine/primaquine regimen according to *ABCB1* haplotypes. Generalized Estimating Equations method with age, gender, co-medication, parasitemia baseline level and genetic ancestry as co-variates; Gene*time $P_{Bonferroni}$ = 0.03 and d = 0.48.

Figure 2. Effect in mean parasitemia reduction from baseline during choloroquine/primaquine regimen comparing *ABCB1* haplotypes. Generalized Estimating Equations method. Day 1 p = 0.5; day 2 p = 0003; day 3 p = 0.2 and day 4 p = 0.3.

Figure 3. Mean parasitemia level reduction during choloroquine/primaquine regimen according to *SLCO2B1* genotypes. Generalized Estimating Equations method with age, gender, co-medication, parasitemia baseline level and genetic ancestry as co-variates; Gene*time $P_{Bonferroni}$ < 0.001 and d = 0.89.

Figure 4. Effect in mean parasitemia reduction from baseline during choloroquine/primaquine regimen comparing *SCLO2B1* genotypes. Generalized Estimating Equations method. Day 1 p = ns ; day 2 AA vs AG p =0.07, AA vs GG p = 0.001, AG vs GG p = 0.2; day 3 p = ns; and day 4 p = ns.

Figure 5. Mean gametocytemia level reduction during choloroquine/primaquine regimen according to *SLCO1B1* haplotypes. Generalized Estimating Equations method with age, gender, co-medication, gametocytemia baseline level and genetic ancestry as co-variates; Gene*time $P_{Bonferroni}$ = 0.04 and d = 1.20.

Figure 6. Effect in mean gemetocytemia reduction from baseline during choloroquine/primaquine regimen comparing *SLCO1B1* haplotypes. Generalized Estimating Equations method. Day 1 p = ns; day 2 *14 vs *1A p = <0.001, *14 vs *1B p = 0.002, *14 vs *15 p = 0.3, *15 vs *1A p = 0.05, *15 vs *1B p = 0.3; and day 3 p = ns.

Figure 1

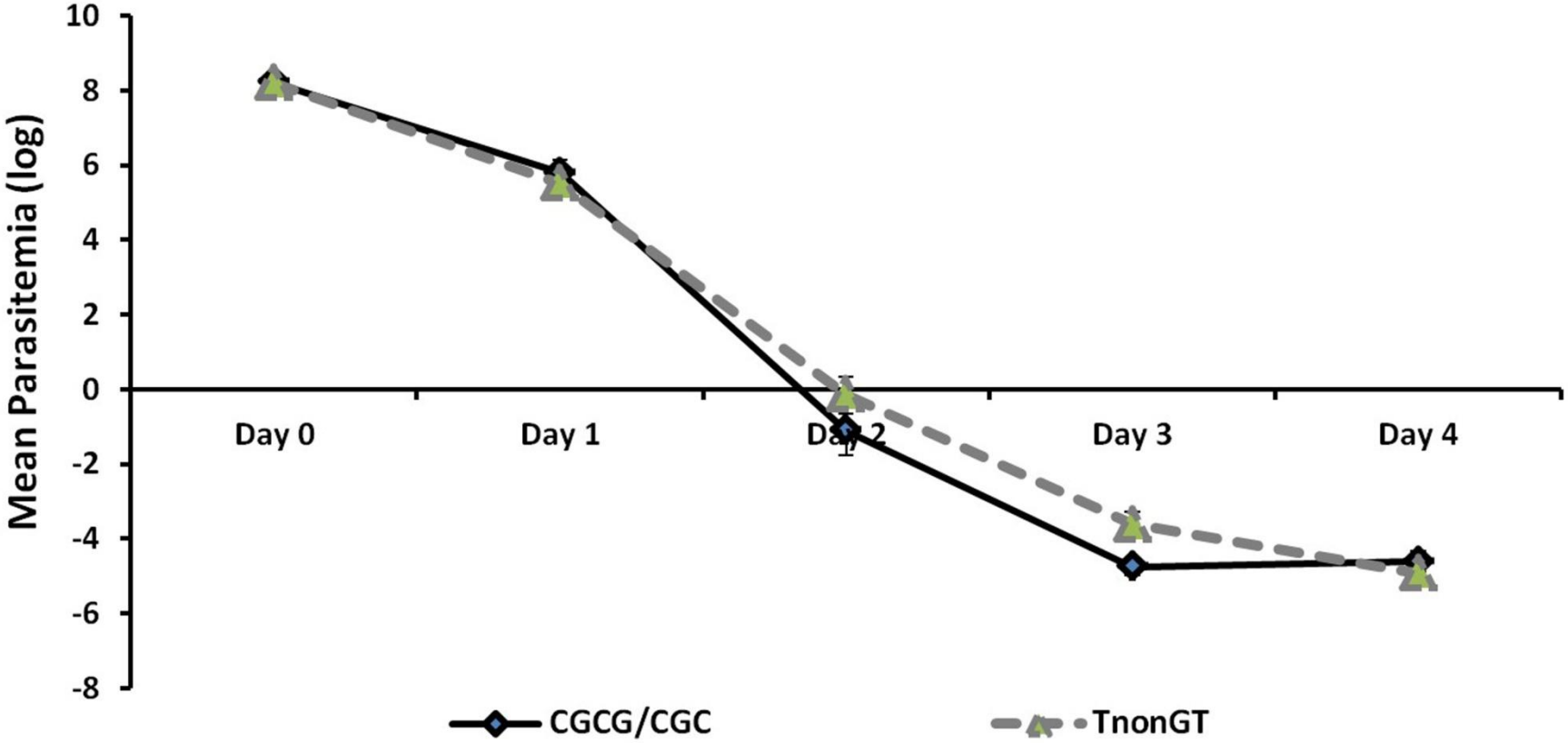

10
8
6
4
2
0
-2
-4
-6
-8
Mean Parasitemia (log)
Day 0
Day 1
Day 2
Day 3
Day 4
CGCG/CGC
TnonGT

Figure 2

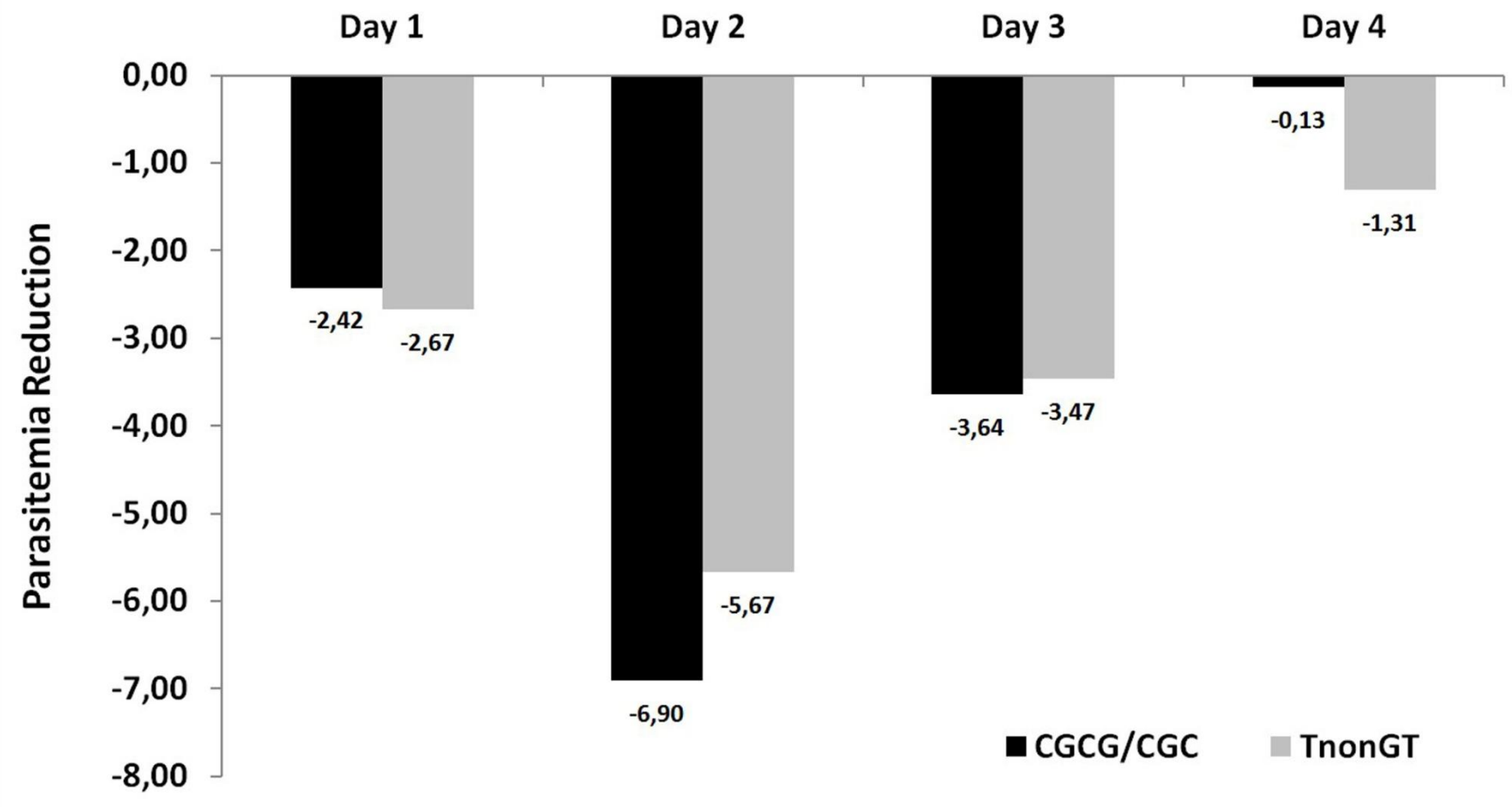

Day 1
Day 2
Day 3
Day 4
0,00
-1,00
-2,00
-3,00
-4,00
-5,00
-6,00
-7,00
-8,00
Parasitemia Reduction
-2,42
-2,67
-6,90
-5,67
-3,64
-3,47
-0,13
-1,31
CGCG/CGC
TnonGT

Figure 3

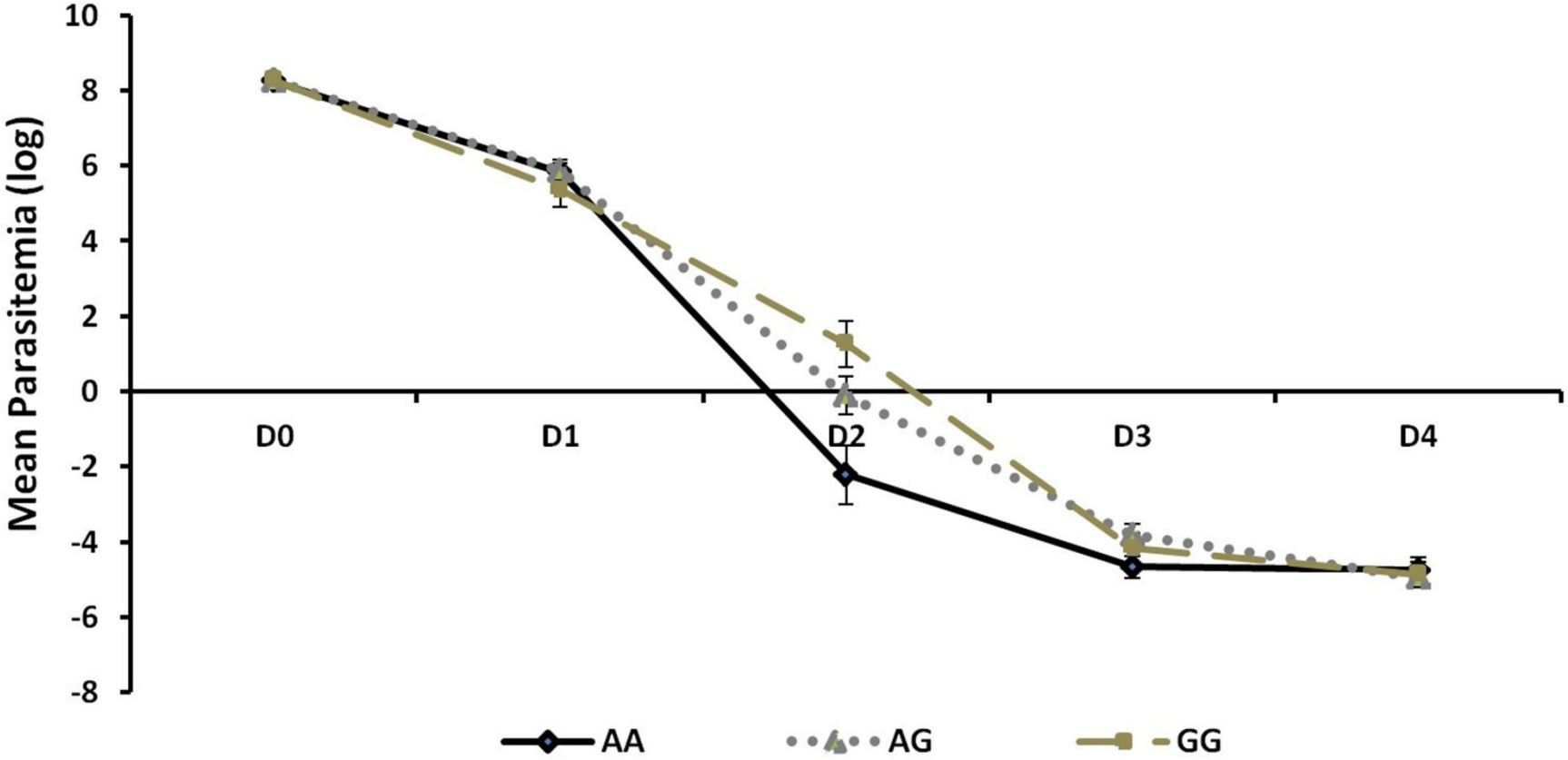
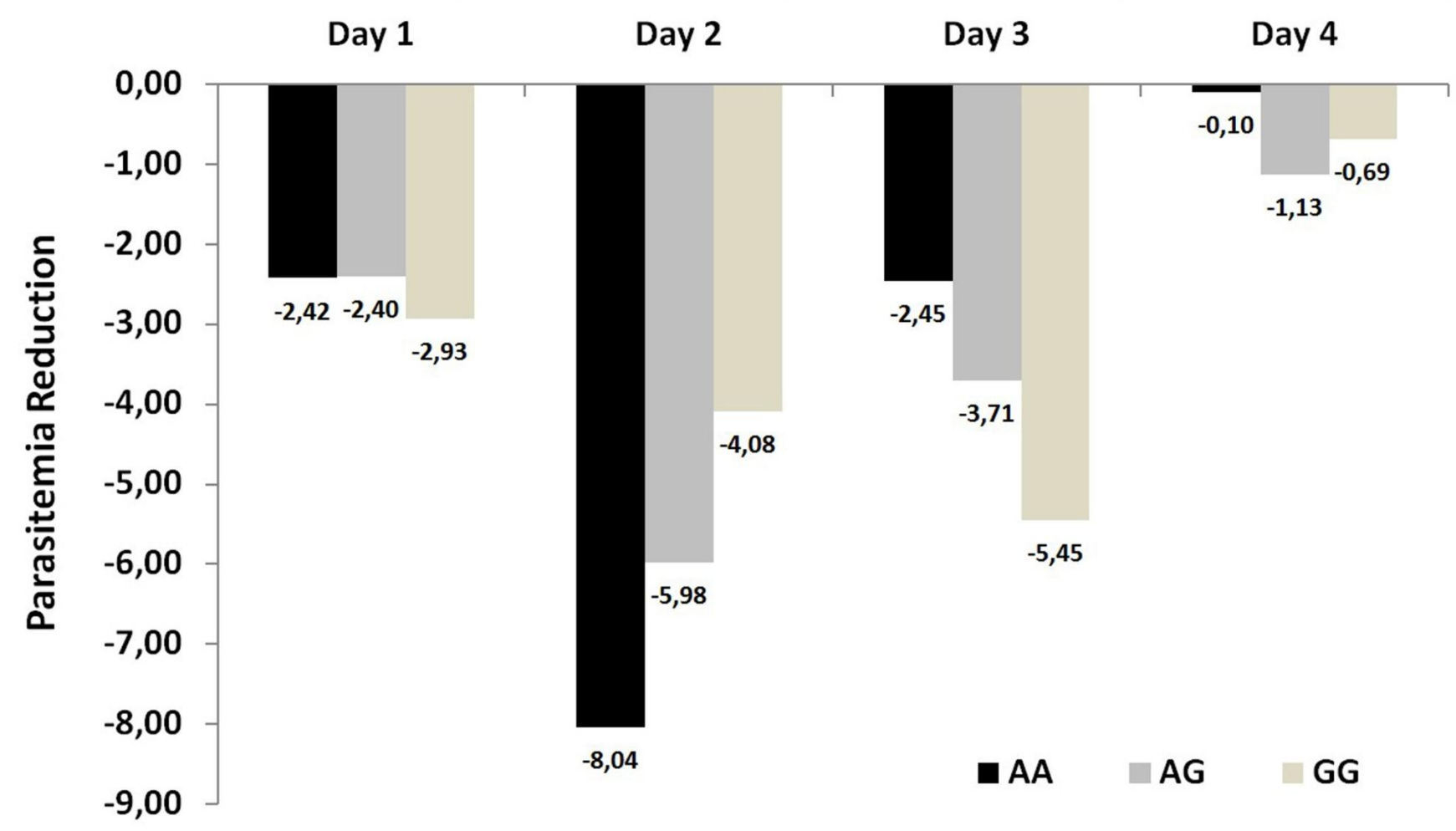


Figure 4


Day 1
Day 2
Day 3
Day 4
Parasitemia Reduction
0,00
-1,00
-2,00
-3,00
-4,00
-5,00
-6,00
-7,00
-8,00
-9,00
-2,42
-2,40
-2,93
-8,04
-5,98
-4,08
-2,45
-3,71
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-0,10
-1,13
-0,69
AA
AG
GG

Figure 5

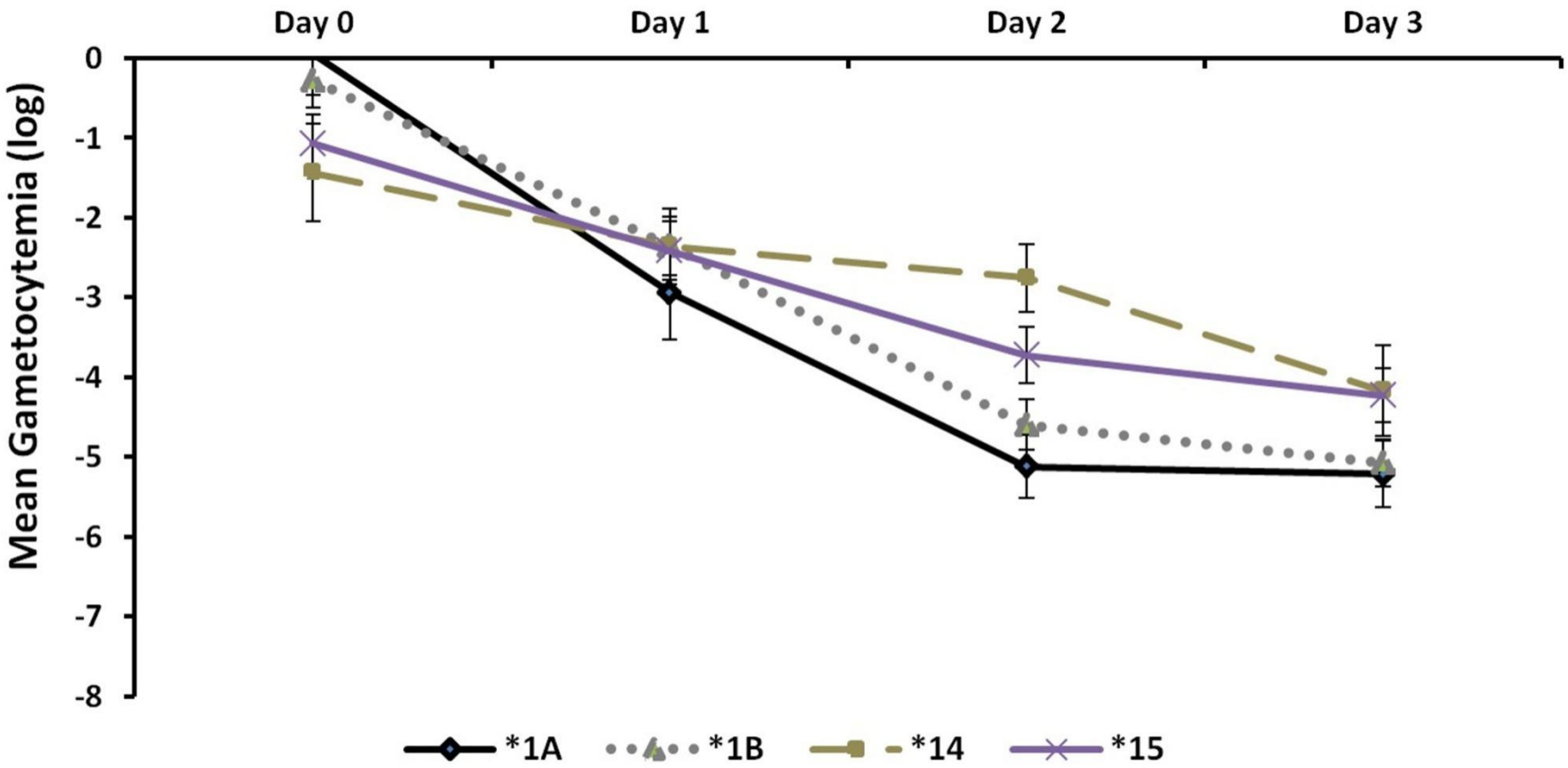
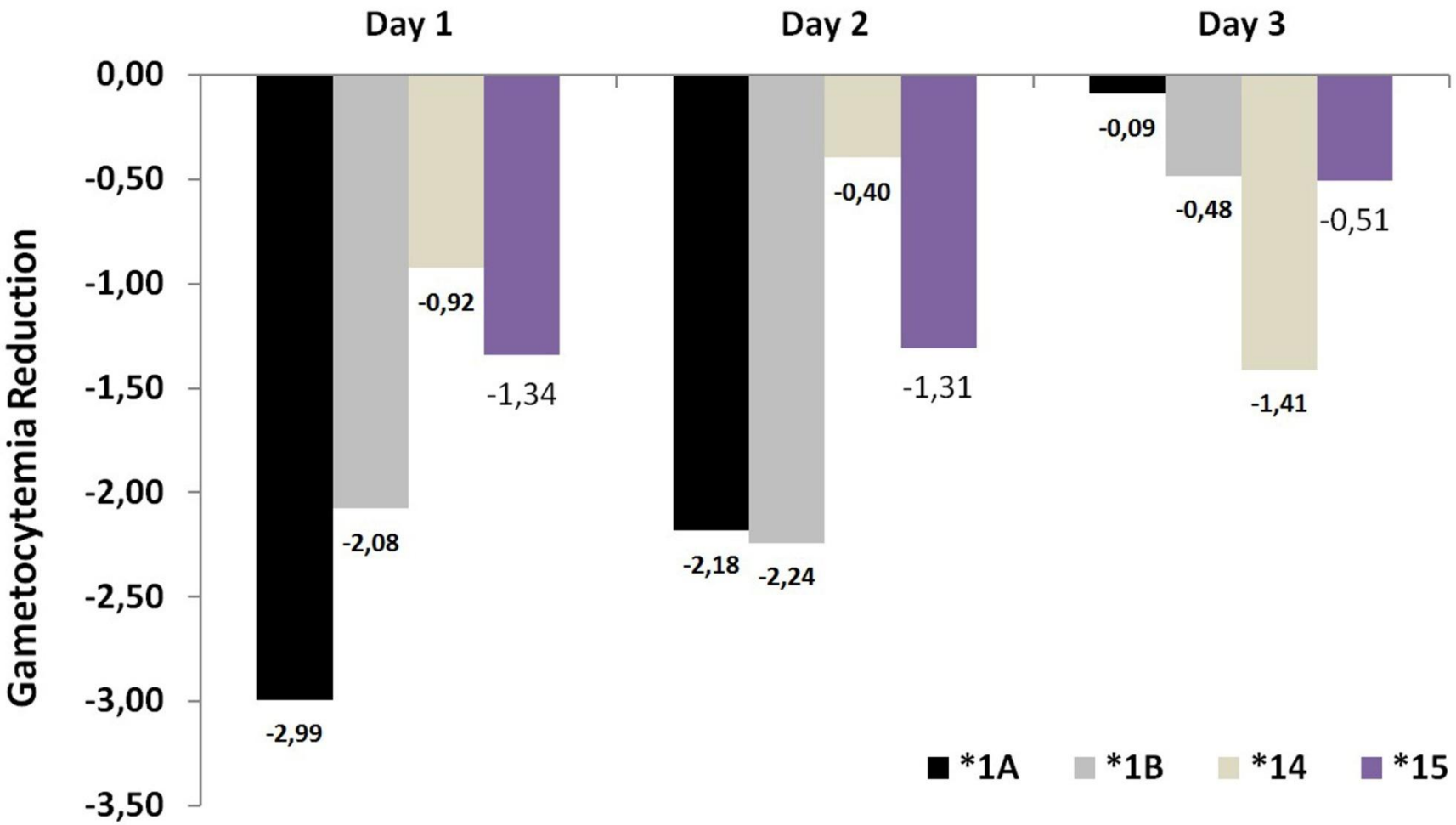

Day 0
Day 1
Day 2
Day 3
Mean Gametocytemia (log)
0
-1
-2
-3
-4
-5
-6
-7
-8
*1A
*1B
*14
*15

Figure 6


Day 1
Day 2
Day 3
Gametocytemia Reduction
0,00
-0,50
-1,00
-1,50
-2,00
-2,50
-3,00
-3,50
-2,99
-2,08
-0,92
-1,34
-2,18
-2,24
-0,40
-1,31
-0,09
-0,48
-1,41
-0,51
*1A
*1B
*14
*15

CAPÍTULO 8
DISCUSSÃO

Considerando-se que os pontos mais específicos dos resultados obtidos já foram discutidos nos capítulos anteriores (3 a 7), neste capítulo final serão abordados aspectos gerais referentes à suscetibilidade às infecções por *P. vivax* e a farmacogenética do tratamento dessas infecções buscando situá-los dentro do contexto atual das pesquisas sobre a malária.

A OMS, a partir do ano 2000, deu início a um novo programa mundial de controle e erradicação da malária. Com a adesão de países de diferentes continentes, os casos dessa doença têm diminuído na última década (World Health Organization, 2012b). As medidas de controle e a utilização de inseticidas tem se mostrado eficientes no combate as infecções por *P. falciparum*, entretanto, por possuir características distintas, as infecções por *P. vivax* tornaram-se um desafio ao esforço mundial de erradicação da malária (Battle *et al*., 2012). As infecções por *P. vivax* muitas vezes são assintomáticas, ou apresentam sintomas muitos dias após o desenvolvimento de gametócitos no sangue do hospedeiro, o que aumenta a chance de transmissão aos mosquitos e a dispersão da doença antes do hospedeiro humano ser tratado (Mendis *et al*., 2001; McKenzie *et al*., 2002). Os gametócitos dessa espécie são transmitidos aos mosquitos de maneira mais eficiente que os do *P. falciparum*, mesmo em pacientes que apresentam um nível de parasitemia baixo (Sattabonkot *et al*., 2004). O *P. vivax* possui a capacidade de permanecer em latência na forma de hipnozoítos e causar recaídas meses após a infecção inicial (James, 1931; Prudencio *et al*., 2006; White, 2011). Somente nos últimos anos, com o reconhecimento de uma maior frequência de casos severos de malária causados por essa espécie de parasito, a comunidade científica começou a ampliar os esforços para a compreensão da distribuição, patofisiologia e suscetibilidade a essa doença.

Os estudos de suscetibilidade genética à malária ou da interação entre patógeno e hospedeiro originalmente foram voltados para compreensão da interação entre os receptores celulares do hospedeiro utilizados pelas espécies de *Plasmodium* para a infecção dos eritrócitos. O receptor celular Duffy é o receptor preferencial utilizado pelo *P. vivax* para a infecção dos eritrócitos sendo um fator determinante para a suscetibilidade a essa doença. Por muito tempo acreditou-se que apenas pessoas Duffy positivos eram suscetíveis a essas infecções, entretanto, na África, onde indivíduos Duffy negativos são predominantes, o *P. vivax* consegue utilizar outros receptores celulares para a infecção dessas células (King *et al*., 2011; Howes *et al*., 2011; Zimmerman *et al*., 2013).

Estudos investigando a resposta imunológica contra a malária geralmente estão voltados para a capacidade de imunização de proteínas do parasito e o desenvolvimento de vacinas (Thakur *et al*., 2008; Butler *et al*., 2012; Thakur *et al*., 2012). Atualmente, trabalhos sobre a regulação e resposta imunológica começam a tomar uma maior importância, embora pesquisas de variantes genéticas no sistema imunológico do hospedeiro e a suscetibilidade à malária causada por *P. vivax* ainda sejam escassos. Nosso trabalho demonstrou que polimorfismos em genes envolvidos no controle e regulação do sistema imunológico estão associados à suscetibilidade, aos níveis de parasitemia e à intensidade dos sintomas da malária. Nossos resultados ressaltam a importância da variabilidade genética do hospedeiro no curso dessas infecções por *P. vivax*, e servem de subsídios para investigações futuras em diferentes populações de áreas endêmicas.

Os fármacos cloroquina e primaquina são utilizados desde a década de 1950 no tratamento da malária, mas, ainda hoje, os mecanismos de absorção, metabolização, excreção e ação desses fármacos não foram completamente descritos. A cloroquina e primaquina são prescritas e utilizadas para o tratamento das infecções de *P. vivax*, *P. ovale* e *P. malariae*, e apresentam variações quanto a sua eficácia em diferentes regiões. Na presente tese, apresentamos os primeiros dados obtidos em uma pesquisa de farmacogenética na utilização desses fármacos no tratamento da malária causada por *P. vivax*. Os resultados apresentados demonstram que variantes genéticas envolvidas na metabolização e no transporte desses fármacos influenciam a velocidade de resposta ao tratamento. Essa variação da resposta pode ter consequências no aparecimento de efeitos adversos e ainda no surgimento de resistência a esses fármacos. O nosso trabalho indica que pacientes que respondem mais rápido ao tratamento podem estar tomando uma dose de fármaco maior do que a necessária, se expondo a um maior risco de apresentar efeitos adversos, enquanto os pacientes que respondem mais lentamente ao tratamento podem receber doses subterapêuticas, aumentando a chance de recaídas e proporcionando o aparecimento da resistência a esses fármacos.

A farmacogenética tem por objetivo a individualização dos tratamentos farmacológicos, potencializando a eficácia e a segurança na utilização de fármacos. No tratamento de doenças infecciosas, a farmacogenética pode ajudar no desenvolvimento de esquemas terapêuticos mais adequados para minimizar o surgimento de cepas resistentes desses parasitos e aumentar a sua eficácia.

A malária no território brasileiro apresenta características singulares, com uma região endêmica praticamente restrita a floresta tropical, que possui um clima favorável para transmissão da doença. Grande variabilidade genética do hospedeiro decorrente da miscigenação entre africanos, ameríndios e europeus (Santos *et al*., 2010) e de espécimes de *P. vivax* com maior variabilidade genética do que a de países da Ásia (Brito e Ferreira, 2011) aumentam a complexidade da investigação dessa doença. Estudos realizados nessa região possuem um grande potencial para auxiliar a compreensão dos fatores genéticos que influenciam tanto a resposta imunológica quanto a farmacogenética do tratamento à malária.

Nosso trabalho apresenta resultados iniciais em uma coorte de pacientes que foi acompanhada e avaliada durante o período do tratamento. No Brasil, a malária ocorre em uma região com uma densidade demográfica baixa, e muitas vezes os pacientes precisam se deslocar de áreas remotas para receber o tratamento. Após o diagnóstico, o paciente recebe os fármacos e a indicação do tratamento, devendo retornar para consulta após o período do tratamento. Entretanto, muitas vezes o paciente só retorna se continua apresentando os sintomas. O acompanhamento durante o período de tratamento e a evolução da doença é difícil de ser realizado, porém estudos longitudinais com o acompanhamento dos pacientes por longos períodos são importantes para conseguir replicar os nossos resultados e avaliar a eficácia do tratamento em relação às recaídas dos pacientes e o aparecimento de efeitos adversos.

O controle e erradicação da malária nos próximos anos passarão pelo desafio do controle das infecções por *P. vivax*. Enquanto a comunidade científica tenta desenvolver uma vacina eficaz contra essa doença que aflige a população humana há muitos anos, estudos de suscetibilidade genética e farmacogenética são ferramentas de grande valor para conseguirmos entender as idiossincrasias dessas infecções em diferentes regiões e fornecer subsídios para aperfeiçoar o controle dessa doença.

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ANEXOS

Universidade Federal de Pará

CNPq-SEPPE Fls. Nº 70

COMITÊ DE ÉTICA EM PESQUISA EM SERES HUMANOS DO CENTRO DE CIÊNCIAS DA SAÚDE DA UNIVERSIDADE FEDERAL DO PARÁ

Carta: 067/07 CEP-CCS/UFPA

Belém, 28 de junho de 2007.

AO: Prof. Dr. João Farias Guerreiro.

Senhor Pesquisador,

Temos a satisfação de informar que seu projeto de pesquisa intitulado: **"Suscetibilidade genética e farmacogenômica e doenças negligenciadas na Amazônia: Malária, Hanseníase e Tuberculose"**, sob o protocolo **nº 061/07 CEP-CCS/UFPA**, foi apreciado e aprovado pelo Comitê de Ética em Pesquisa em Seres Humanos do Centro de Ciências da Saúde da Universidade Federal do Pará na reunião do dia 13 de junho de 2007.

Assim, Vossa Senhoria tem o compromisso de entregar o relatório do mesmo até o dia 30 de dezembro de 2007, no CEP-CCS/UFPA, situado no Campus Universitário do Guamá, Campus profissional, no Complexo de sala de aula do CCS – sala 13 (Altos).

Atenciosamente,

Profª. *M. Sc.* Maria da Conceição S. Fernandes.
Coordenadora do CEP-CCS/UFPA

Comitê de Ética em Pesquisa em Seres Humanos do Centro de Ciências da Saúde da Universidade Federal do Pará (CEP-CCS/UFPA) - Complexo de Sala de Aula/ CCS - Sala 13 - Campus Universitário do Guamá, nº 01, Guamá -- CEP: 66075-110 - Belém-Pará. Tel./Fax. 3201-8028/3201-7735 E-mail: cepccs@ufpa.br

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PLoS one

The Genomic Ancestry of Individuals from Different Geographical Regions of Brazil Is More Uniform Than Expected

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Abstract

Based on pre-DNA racial/color methodology, clinical and pharmacological trials have traditionally considered the different geographical regions of Brazil as being very heterogeneous. We wished to ascertain how such diversity of regional color categories correlated with ancestry. Using a panel of 40 validated ancestry-informative insertion-deletion DNA polymorphisms we estimated individually the European, African and Amerindian ancestry components of 934 self-categorized White, Brown or Black Brazilians from the four most populous regions of the Country. We unraveled great ancestral diversity between and within the different regions. Especially, color categories in the northern part of Brazil diverged significantly in their ancestry proportions from their counterparts in the southern part of the Country, indicating that diverse regional semantics were being used in the self-classification as White, Brown or Black. To circumvent these regional subjective differences in color perception, we estimated the general ancestry proportions of each of the four regions in a form independent of color considerations. For that, we multiplied the proportions of a given ancestry in a given color category by the official census information about the proportion of that color category in the specific region, to arrive at a "total ancestry" estimate. Once such a calculation was performed, there emerged a much higher level of uniformity than previously expected. In all regions studied, the European ancestry was predominant, with proportions ranging from 60.6% in the Northeast to 77.7% in the South. We propose that the immigration of six million Europeans to Brazil in the 19th and 20th centuries - a phenomenon described and intended as the "whitening of Brazil" - is in large part responsible for dissipating previous ancestry dissimilarities that reflected region-specific population histories. These findings, of both clinical and sociological importance for Brazil, should also be relevant to other countries with ancestrally admixed populations.

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Introduction

Continental populations of the world vary considerably in their predisposition to diseases and in the allele frequencies of important pharmacogenetic loci, probably as a result of genetic drift, but also because of adaptation to local selective factors such as climate and available nutrients. In many countries, skin color has traditionally been used in clinical and pharmacological studies as a phenotypic proxy for geographical ancestry. Brazil is no exception.

The Brazilian population was formed by extensive admixture from three different ancestral roots: Amerindians, Europeans and Africans. This resulted in a great variability of skin pigmentation, with no discontinuities between Black and White. For instance, in a single small fishing village in Brazil, Harris and Kotak [1] identified dozens of designations for varied shades of skin pigmentation.

However, the Instituto Brasileiro de Geografia e Estatística (IBGE), which is responsible for the official census of Brazil, has employed only few pre-established color categories, which are based on self-classification. Since 1991 they number five: White ("branca"), Brown ("parda"), Black ("preta"), Yellow (amarela) and Indigenous ("indígena"). Brown ("pardo") emerged as a

synthesis of a variety of classifications, such as "caboclo", "mulato", "moreno", "cafuzo", and other denominations that express the admixed character of the Brazilian population [2].

In general, there is academic support for the IBGE classification system, which is the only source of information on color categories at a national level [3,4]. It reflects the fact that in Brazil social "racial" categorization depends not on ancestry, but on the physical appearance of the individual [5].

In 2008 IBGE ascertained a population of *circa* 190 million Brazilians who, based on self-classification, could be segregated into the following proportions for color: 48.4% White, 43.8% Brown, 6.8% Black, 0.6% Yellow, 0.3% Indigenous and 0.1% with no declaration (http://www.sidra.ibge.gov.br/bda/tabela/listabl.asp?z = t&c = 262). The first three of these categories (White, Brown and Black) encompass 99.1% of the Brazilian population and will be the focus of this study. It is important to realize that in Brazil, color (in Portuguese, *cor*) denotes the Brazilian equivalent of the English term race (*raça*) and is based on a complex subjective phenotypic evaluation that takes into account, not only skin pigmentation, but also hair pigmentation and type, eye melanization and facial features such as nose and lip shape [6].

With an area of 8,511,960 Km^2, Brazil has a territory of continental size (the fifth largest in the world) and different regions have diverse population histories. For instance, the North had a large influence of the Amerindian root, the Northeast had a history of strong African presence due to slavery and the South was mostly settled by European immigrants. These different compositions were quite evident in our studies of mtDNA haplotypes of White Brazilians [7,8].

When we look into the Brazilian census data on the proportion of each color category according to region, we indeed can see noticeable differences (Table 1). In the North and Northeast there is a strong predominance of Brown individuals (64.0% and 58.0%, respectively) while in the Southeast and South, White Brazilians constitute the largest category (62.4% and 83.6%, respectively). The Center-West, the least populous region, includes the heterogeneous Federal District (i.e. Brasilia) and displays more even proportions of White and Brown individuals (49.7% and 43.7%, respectively). Such high level of regional structure is peculiar, especially when we consider that our previous work has shown only a feeble relationship between color and ancestry in Brazilians [8–10].

We have already shown that a set of 40 short insertion-deletion (indel) polymorphisms was sufficient for an adequate characterization of human population structure at the global level [11]. We furthermore demonstrated the resolution power of these markers in discriminating among Europeans, Africans and Amerindians by plotting in a triangular graph our results with the samples of Europeans, Africans and Amerindians of the HGDP-CEPH Diversity Panel [12]. Three totally divergent clusters that correspond to the European, African and Amerindian populations were obtained without any overlap — each group clustered in one of the vertices of the triangular plot [8].

In the present study we used these loci to estimate the Amerindian, European and African genomic ancestry of 934 Brazilians from the four most populous geographical regions of the Country, self-categorized as White, Brown and Black.

Results

Estimates of the trihybrid ancestry of Brazilians from different regions

In the present work we established the genotype of 934 self-classified White, Brown or Black Brazilians at 40 autosomal short insertion-deletion polymorphisms (indels) dispersed in the human genome. The allele frequencies at these loci are shown in Table S1. We then used the genotypes and the *Structure* program to estimate, at an individual level, the European, African and Amerindian components of ancestry for these individuals from states in four geographical regions of Brazil (Fig. 1). All the individual estimates are shown in triangular plots in Fig. 2. We then calculated the mean and standard error of individual estimates to arrive at a summarizing figure for each group (Table 2).

Table 1. 2008 IBGE data for the regions and states sampled in this study.

		Population	White	Brown	Black
		(X 10^3)			
Brazil		**189,953**	**92,003**	**83,196**	**12,987**
			(48.43%)	**(43.80%)**	**(6.84%)**
Region	**State**				
North	Pará	7,367	1,530	5,374	398
		(3.88%)	(20.77%)	(72.95%)	(5.40%)
	Ceará	8,472	2,800	5,370	257
Northeast		(4.46%)	(33.05%)	(6339	(303
	Bahia	14,560	2,999	9,149	2,328
		(7.67%)	(20.60%)	(62.84%)	(15.99%)
Southeast	Rio de Janeiro	16,203	8,509	5,302	2,328
		(8.53%)	(52.51%)	(32.72%)	(14.37%)
	Santa Catarina	6,091	5,297	608	160
South		(3.21%)	(86.96%)	(9.98%)	(2.63%)
	Rio Grande	10,856	8,776	1,495	529
	do Sul	(5.72%)	(80.84%)	(13.77%)	(4.87%)

The first column shows the total population of Brazil and the population of each state expressed in absolute values and percentage of the total for the whole Country. The columns for the color categories contain data also expressed in absolute numbers and percentages self-categorized in that region (in parentheses). The percentages for Whites, Blacks and Browns do not add to 100% because each State has individuals who belong to color categories that are distinct from the ones shown. Data obtained from http://www.sidra.ibge.gov.br/bda/tabela/listabl.asp?z = t&c = 262.

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The most evident diversity in the ancestral Amerindian, European and African proportions of the different color categories, both between and within the different regions of Brazil, was seen in individuals self-assessed as Brown. For instance, in the North (Belém, PA) self-classified Brown individuals had, on the average, 68.6% European ancestry, followed by 20.9% Amerindian ancestry and 10.6% African ancestry, while in the South they had, on the average, 44.2% European, 11.4% Amerindian and 44.4% African ancestries.

To estimate the significance of the pairwise differences observed between the samples of individuals self-classified as Brown in diverse regions, we used a specially designed Monte Carlo randomization test of the distance D between the means, described in detail in the Material and Methods section. In Table S2 the observed distances are in the cells above the diagonal and the probability of obtaining the observed distances by chance is shown in the cells below the diagonal.

Since we have six comparisons, we need to control for type I error. Applying the Bonferroni correction [13,14] the alpha value

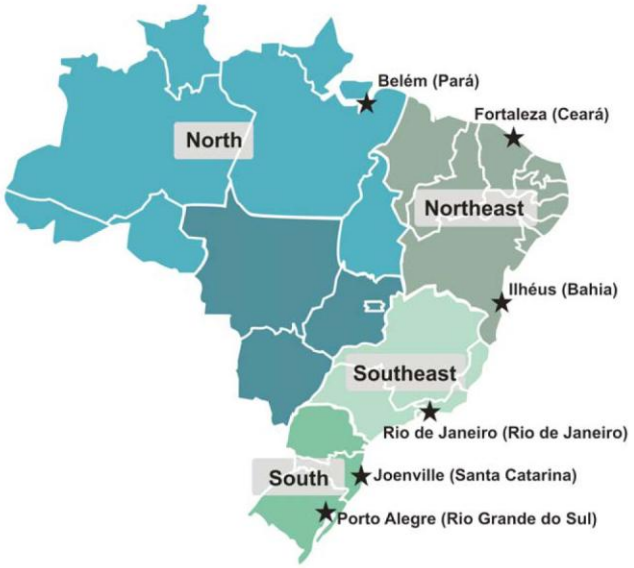

Figure 1. Map of Brazil showing the five geographical regions of the country. The regions with a square label were analyzed in this work. The cities and respective states where the samples were collected are shown with a star.
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for significance was reduced to 0.008. Even then, four of the six contrasts are significant, i.e. between North (Pará) and Southeast (Rio de Janeiro); between North (Pará) and South (Rio Grande do Sul); between the Northeast (Ceará) and the South (Rio Grande do Sul) and between the Southeast (Rio de Janeiro) and the South (Rio Grande do Sul). This reinforces the idea that the semantic criteria for color self-categorization are heterogeneous in the different Brazilian regions.

Estimates of the "total ancestry" of different regions of Brazil

Since both the census proportions of each color category and the trihybrid ancestry of Brazilians vary according to region, we decided to merge the two sets of data and estimate what we have called the "total ancestry" of a given region. This has the advantage of circumventing the different regional semantics of what it means "to be" White, Brown or Black. To calculate the total ancestry we simply multiply the proportions of a given ancestry in a given color category by the census proportion of that color category in the specific region to arrive at an ancestry estimation regardless of color.

In order to show how the calculation of the "total ancestry" was done, let us take the example of European ancestry in the North region (state of Pará) using the data from Table 2. In that state, White, Brown and Black individuals have average European ancestry of 0.782, 0.686 and. 0.524 respectively. Since for the state of Pará the census shows the relative proportions of the same three colors above as 0.210, 0.736 and 0.055, the weighted European ancestry, which is now independent of color, will be $(0.782 \times 0.210) + (0.686 \times 0.736) + (0.524 \times 0.055) = 0.697$.

The "total ancestry" estimates thus calculated for all regions are shown in the rightmost column of Table 2. The calculation could not be performed for two of the samples, Ceará and Santa Catarina, because they lacked data on one or more color categories.

The results obtained showed that there is in fact a smaller level of variability between the different regions than had been observed in the census data of color categories or in the ancestry proportions of the different color classes (Fig. 1). In all regions studied the European ancestry surfaced as uniformly preponderant, with proportions of 69.7%, 60.6%, 73.7% and 77.7%, respectively (Table2). This suggests that the populations of different regions of Brazil are ancestrally more similar than previously realized.

Discussion

The trihybrid ancestry of Brazilians from different regions

We here present results of the molecular estimation of the European, African, and Amerindian ancestry in 934 individuals belonging to different color categories and originated from four regions of Brazil (Figure 1). The picture that emerges from the data is of great heterogeneity both within and between color categories and geographical regions (Figure 2 and Table 2). A noteworthy observation is that there is considerable admixture, which can be appreciated both at the individual and the group level. Individually, triangular plots show great variation in ancestry levels for all color categories and in all regions (Fig. 1). At a group level, for instance, we can observe that the African ancestry of Black individuals is below 50% in all samples tested, with the exception of the state of Santa Catarina, in the South.

In a previous publication [9], with different samples, a weak relationship had already perceived between color and geographic genomic ancestry in Brazilians at an individual level, as we also observed in the present study. If we consider some peculiarities of Brazilian history and social structure, we can understand why indeed this should be so. Africans characteristically have black skin associated with other iconic individual components of color (black curly hair, black eyes, broad nose and thicker lips) and Europeans have white skin associated iconic individual components (light straight hair, light-colored eyes and thin nose and lips), all genetically determined by a relatively small number of genes that were evolutionarily selected by the geographical environment, especially the prevailing levels of sun UV exposure [15]. Thus, if we have a social race identification system based primarily on phenotype, such as occurs in Brazil, we classify individuals on the basis only of the presence of certain alleles at a relatively small number of genes that have impact on color, while ignoring the rest of the genome (where the 40 indels that we used to estimate ancestry are located). Now, if we have a population that is produced by extensive admixture of Europeans and Africans, as we know happened in Brazil, the association between ancestry and color should dissipate with time, which is exactly what we have seen.

The heterogeneity in the ancestry estimates of different color categories in different regions

Another important observation is the considerable variability in the ancestry of color categories in different regions, most manifest in Brown and Black individuals. For instance, self-classified Brown individuals from the North had on the average 68.6% European ancestry, while in the South they had on the average 44.4% African ancestry. Also, for individuals self-classified as Black we can see considerable, but highly discrepant levels of European ancestry varying from 29.3% in Santa Catarina to 53.9% in Bahia. The most uniform category was that of individuals self-classified as White who consistently had a predominant European ancestry, varying from 66.8% in Bahia (BA) to 85.5% in Rio Grande do Sul and 86.1% in Rio de Janeiro.

It is noteworthy that such different regional subjective differences in color perception unraveled by our ancestry analysis appear to run counter to expectations based on pre-genomic

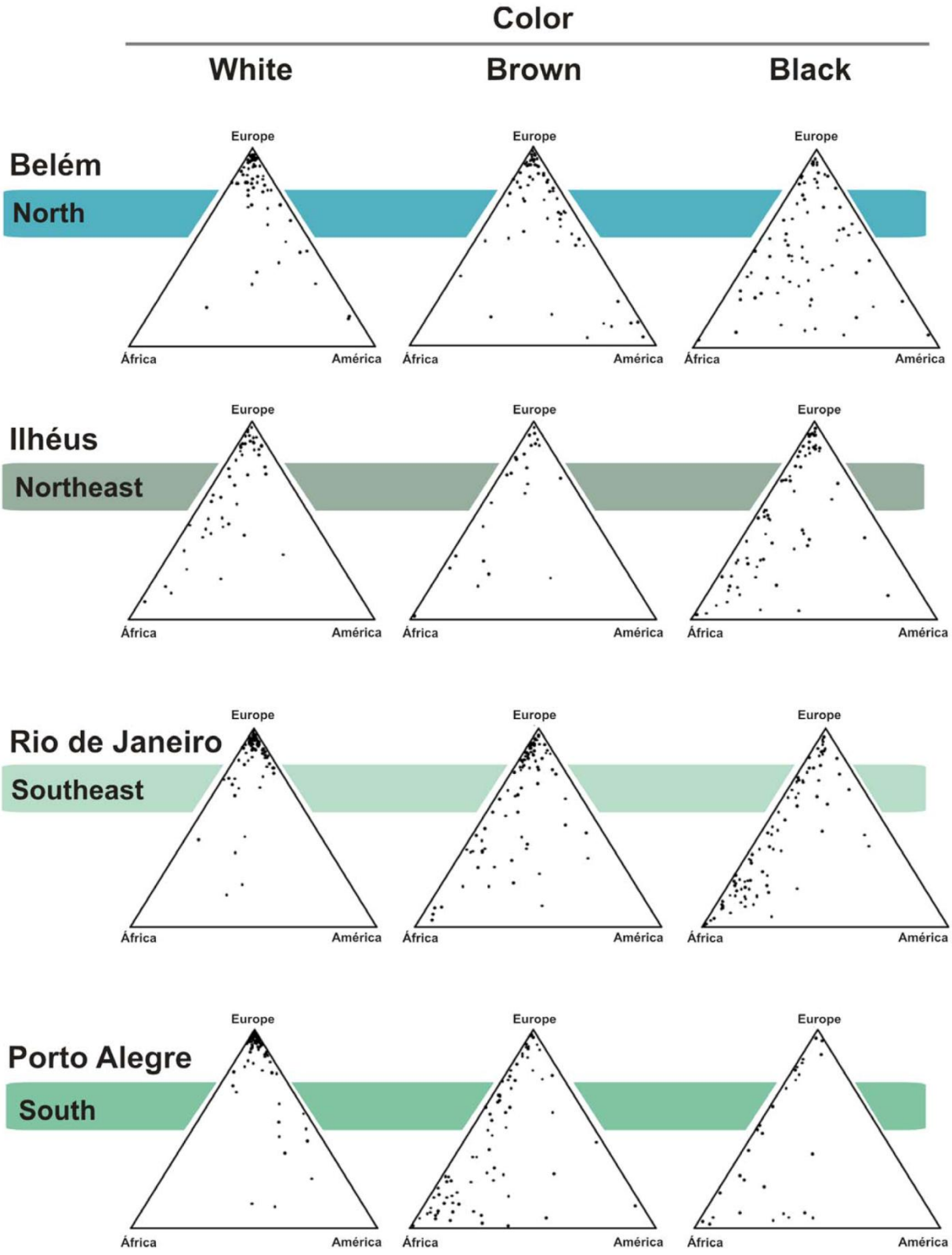

Figure 2. Triangular plots of the genomic proportions of African, European and Amerindian ancestry in three self-reported color groups of 934 Brazilian individuals from four different regions of the country, self-categorized White, Brown and Black individuals. Each point represents a separate individual and the ancestral proportions can be determined by dropping a line parallel to each of the three axes. The graphs were drawn using the Tri-Plot program [42].
doi:10.1371/journal.pone.0017063.g002

Table 2. Mean and standard error for the estimated Amerindian, European and African ancestries of 934 Brazilian individuals from four regions of the country, self-categorized as having White, Brown and Black color.

Region	Ancestral Roots	Color category						Color-independent "Total Ancestry"
		White		Brown		Black		
		Mean	s.e.	Mean	s.e.	Mean	s.e.	
North	European	0.782	0.026	0.686	0.034	0.524	0.031	0.697
(Pará)	African	0.077	0.011	0.106	0.016	0.275	0.023	0.109
	Amerindian	0.141	0.022	0.209	0.030	0.201	0.026	0.194
Northeast	European	0.668	0.037	0.603	0.060	0.539	0.034	0.606
(Bahia)	African	0.244	0.033	0.308	0.057	0.359	0.014	0.303
	Amerindian	0.088	0.012	0.089	0.020	0.101	0.031	0.091
Northeast	European	0.758	0.032	0.728	0.029	N.S.	N.S.	
(Ceará)	African	0.133	0.017	0.144	0.021	N.S.	N.S.	
	Amerindian	0.109	0.021	0.128	0.015	N.S.	N.S.	
Southeast	European	0.861	0.016	0.675	0.028	0.427	0.032	0.737
(Rio de Janeiro)	African	0.074	0.011	0.238	0.025	0.495	0.032	0.189
	Amerindian	0.065	0.007	0.087	0.012	0.079	0.009	0.074
South	European	0.855	0.021	0.442	0.037	0.431	0.062	0.777
(Rio Grande do Sul)	African	0.053	0.019	0.444	0.035	0.459	0.052	0.127
	Amerindian	0.093	0.006	0.114	0.016	0.110	0.026	0.096
South	European	N.S.	N.S.	N.S.	N.S.	0.293	0.031	
(Santa Catarina)	African	N.S.	N.S.	N.S.	N.S.	0.596	0.030	
	Amerindian	N.S.	N.S.	N.S.	N.S.	0.111	0.012	

N.S. = Not studied.

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racial/color methodology. For instance, Osorio [4] discusses a hypothetical case of twins with identical ancestry proportions and with phenotypes in the White-Brown frontier and who have been raised separately, one in Bahia and the other in South Brazil. He then states that it would be expected that the one in Bahia should be considered White while the other, in the South, would be considered Brown or Black. But what our data shows is the opposite: Browns and Black individuals in Bahia have in fact less African ancestry than their counterparts in the South.

One possible explanation for this might be the effect of darker pigmentation by sun exposure. Jablonski and Chaplin [16] have shown that skin reflectance is strongly correlated with absolute latitude and UV radiation levels. This is due in large part to environmental factors, i.e., UV exposure [15]. Brazil occupies a subtropical position and the UV exposure in the North and Northeast is higher than in the Southeast and considerably higher than in the South. Thus, it might be necessary to have a higher level of African Ancestry present to have a Brown or Black skin color in the South, with lesser sun exposure, than in the northern regions of Brazil. A second, additive, possibility is that there are different cultural semantic criteria for color classification in diverse regions.

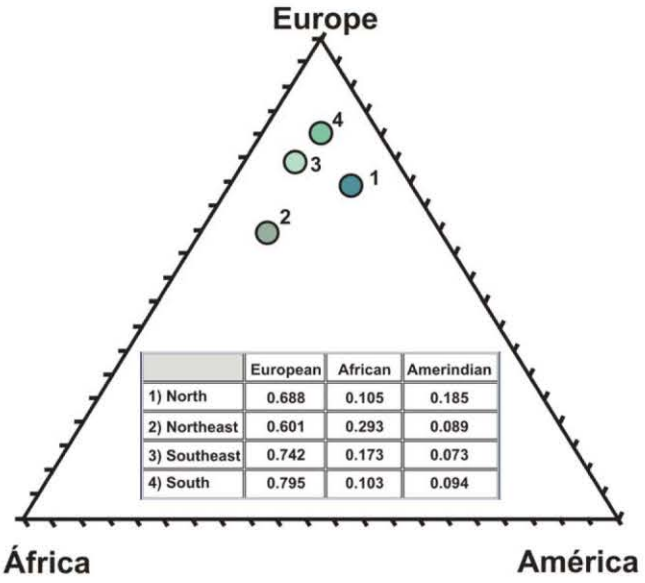
Independent of the reason, it is evident that ancestrally people who are White, Brown or Black in the northern part of Brazil are different from their counterparts in the southern part of the Country. This shows that, as has been pointed out before [9], the relationship between color and geographical ancestry is tenuous and one cannot use interchangeably terms such as White, Caucasian and European in one hand, and Black, Negro or African in the other, as is often done in daily discourse, in political rhetoric and in the medical and scientific literature.

Total ancestries

To eschew the use of color categories we decided to try to estimate the general ancestry proportions of the different regional samples independent of color categories. To do that, we multiplied the proportions of a given ancestry in a given color category by the census proportion of that color category in the specific region, to arrive at ancestry estimation independent of color. Once such a correction was performed on the basis of the relative proportion of Amerindian, European and African ancestries, there emerged a higher level of uniformity than expected. In all regions studied the European ancestry was predominant, with proportions being ranging from 60.6% in the Northeast to 77.7% in the South (Figure 3). The African proportion was highest in the Northeast (30.3%), followed in decreasing order by the Southeast (18.9%), South (12.7%), and North (10.9%). On the other hand, the Amerindian proportion was highest in the North (19.4%), while relatively uniform in the other three other regions.

This is novel genetic information about the Brazilian people that needs to be placed on a historical and phylogeographical context. First, we will compare them with our previous observations with uniparental genetic markers in Brazilians.

We earlier examined DNA polymorphisms in the non-recombining portion of the Y-chromosome and in the hypervariable region of mitochondrial DNA (mtDNA) in the four main regions of the Country (the same four regions analyzed in the present paper, although with samplings from different states). The vast majority of Y-chromosomes, independent of the region, proved to be of European origin [17,18]. Studies of mtDNA revealed a different reality: considering Brazil as a whole, 33%, 39% and 28% of matrilineages were of Amerindian, European



	European	African	Amerindian
1) North	0.688	0.105	0.185
2) Northeast	0.601	0.293	0.089
3) Southeast	0.742	0.173	0.073
4) South	0.795	0.103	0.094

Figure 3. Triangular plot and table of the genomic proportions of African, European and Amerindian ancestry in four different regions of Brazil, independent of color category. Each point represents a separate region, as follows (1) North (Pará), (2) Northeast (Bahia), (3) Southeast (Rio de Janeiro) and (4) South (Rio Grande do Sul). The graph was drawn using the Tri-Plot program [42].
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and African origin, respectively [7]. Significantly, the frequency of mtDNA ancestries varied a lot in different regions: most matrilineal lineages in the Amazonian region had Amerindian origin (54%), while African ancestry was preponderant in the Northeast (44%) and European haplogroups were prevalent in the South (66%). These data have since been amply confirmed by other studies [8]. Together, they configured a picture of strong directional mating between European males and Amerindian and African females, which agrees perfectly with the known history of the peopling of Brazil since 1500 [8].

The proportions of Amerindian and African maternal ancestry were higher in the previous investigation using mtDNA than in the regional total ancestry averages calculated in the present study using biparental markers. However, it is interesting to note that both studies agree in that the highest level of Amerindian ancestry could be found in the North region (54% for mtDNA; 19.4% in the present study) and the highest level of African ancestry belonged to the Northeast region (44% for mtDNA; 30.3% in the present study), exactly as expected from known historical and anthropological studies of Brazilians [19].

A unifying proposal

As mentioned previously, Brazil is the home of genetically heterogeneous people, the product of five centuries of admixture between Amerindians, Europeans and Africans. However, such admixture has occurred in a sexually asymmetric fashion, as a result of the colonization model employed by the Portuguese. Indeed, we know that few women came from Portugal to Brazil in the period from the arrival of the Europeans in 1500 until 1808, when the Portuguese Court fled the Napoleonic invasion of the Iberian Peninsula and relocated to Rio de Janeiro [20]. That means that the Brazilian population was primarily formed by male Portuguese and female native Amerindian and enslaved Africans.

Initially, the whole population was composed by the indigenous Amerindians. Little is known about their number when the Portuguese arrived in 1500 [21], although a figure often cited is that of 2.5 million individuals [21]. From 1500 to 1808, it is estimated that about 500,000 Portuguese colonizers, almost exclusively men, came to Brazil [20], admixing extensively with the Amerindian women. Thus, we can expect that the first generation of Brazilians genomically had 50% Amerindian and European ancestry, but 100% Amerindian mtDNA. Further generations of admixture with the Portuguese lead to progressive "europeanization" of genomic ancestry, while maintaining an elevated proportion of Amerindian mtDNA.

The slave traffic started in the middle of the 16th century, extending until 1850 and resulting in the forced relocation of an estimated 4 million Africans to Brazil [21]. These three centuries were a period of intense interbreeding between European males and Amerindian and African women, which led to introgression of genomic African ancestry into Brazilians and also of African mtDNA, since the African contribution was primarily from females [7].

Let us take, as a generic example, the mating of a white European male with a Black African slave woman in Brazil. Because of the Brazilian social race identification system based primarily on phenotype, the children with dark skin pigmentation and other African iconic individual components of color would be considered Black, while those with light colored skin and other European iconic individual components of color would be considered White, even though they would have exactly the same proportion of African and European alleles [9]. Since in Brazil there also occurs assortative mating by color (as has indeed been revealed by demographic studies) [22,23], in the hypothetical subsequent generation, the light-skinned individuals would tend to marry other Whites and conversely the dark-skinned individuals would marry Blacks. The long-term tendency would then be for this pattern to produce two distinct color groups, White and Black, which would, nonetheless, both have simultaneously a significant level of European and African ancestry.

It is relevant to notice that 1.72 million slaves (42.9% of the total) arrived in Brazil during the first half of the 19th century, a time by which the number of Amerindians in Brazil had dwindled due to strife and/or European-borne disease. Most likely, the main contribution of Amerindians to the formation of the Brazilian people occurred in the first 2 or at most 3 centuries of its colonization, no longer being of high importance in the early 19th century, when larger and larger portions of Brazilians moved from rural areas to the cities. Since Africans (up until 1850) and Europeans (up until the 20th century) continued to arrive to Brazil and to participate in the gene pool, the Amerindian ancestry component was diluted across color-lines to the levels that we observe presently, but without losing its mtDNA representativity because of the sexual asymmetry of the relationships. The resulting highly admixed Brazilian population can be assessed by the proportions of the color categories in first Brazilian census in 1872, which was 19.7% Black, 42.2% Brown and 38.1% White.

In 1850, the forced arrival of Africans stopped due to prohibition of the slave trade. At the same time the Government started a campaign to stimulate the immigration of Europeans to Brazil. This process, which has been denominated the "Whitening of Brazil" had complex economic and sociological causes, and was tinged with racist ideology [24–27]. In the approximately one hundred year period 1872–1975, Brazil received 5,435,735 million immigrants from Europe and the Middle East [21]. These were, in decreasing percentages, 34% Italians, 29% Portuguese, 14% Spanish, 5% Japanese, 4% Germans, 2% Lebanese and Syrians and 12% others [21].

This huge demographic event is probably responsible for the noteworthy dissipation of previously established regional differ-

ences in ancestries, as the European component of ancestry became uniformly preponderant, with similar proportions of 69.7%, 60.6%, 73.7% and 77.7% in the North, Northeast, Southeast and South, respectively.

How to explain why no similar wash-out occurred in respect to the matrilineal ancestry? We believe that the regional disparities in mtDNA ancestry were maintained because, once again, in the immigratory wave of Europeans there was a significant excess of males. When they admixed with the Brazilian women there was rapid europeanization of the genomic ancestry, but preservation of the established matrilineal pattern. There is demographic information to corroborate this possibility. First, of 1,222,282 immigrants from all origins that arrived in the Port of Santos in the period 1908–1936 the sex ratio (males/females) was 1.76 [28]. Second. the two most abundant immigrants, Portuguese and Italians, had sex rations of 2.12 and 1.83, respectively. census data of 1910 showed concordant results: there were 1,138,582 foreigners in Brazil, with a male/female ratio of 1.74, while there were 22,275,595 Brazilians with an even sex ratio of 1.02 [29].

Clinical Implications

Understanding the heterogeneity and admixture of Brazilians within and between geographical regions has important clinical implications for the design and interpretation of clinical trials, the practice of clinical genetics and genomic medicine, the implementation of pharmacogenetic knowledge in drug prescription, and the extrapolation of data from other, more homogeneous populations.

Let us take the case of VKORC1, a key enzyme of the vitamin K cycle that is a molecular target of the coumarin anticoagulant warfarin. Polymorphisms of the *VKORC1* gene vary markedly in frequency worldwide, and this diversity is a major determinant of the individual dose requirement and clinical response to warfarin and other anticoagulants in several populations [30]. For example, the higher frequency of the warfarin-sensitive *VKORC1* 1173T variant allele in Japanese (0.89) compared to Caucasians (0.42) explained why the median warfarin dose is significantly higher in Caucasian than Japanese patients [31]. Accordingly, Limdi et al [32] have recently shown that differences in the warfarin dose requirements explained by *VKORC1* across several populations worldwide are largely accounted by the minor allele frequency of the *VKORC1* 1173C>T and 3673G>A (alternatively known as -1639G>A) SNPs.

We genotyped the *VKORC1* polymorphisms 3673G>A (rs99232315), 808T>G (rs2884737), 6853G>C (rs8050894) and 9041G>A (rs7294) in the same individuals analyzed in the present article [33]. We then inferred the statistical association between the distribution of the *VKORC1* haplotypes among Brazilians and self-reported color, geographical region and genetic ancestry by fitting multinomial log linear models via neural networks. The frequency distribution of the *VKORC1* haplotypes among Brazilians varied across geographical regions and self-reported color categories. Notably, the frequency of the warfarin sensitive *VKORC1* 3673A allele and the distribution of *VKORC1* haplotypes varied continuously as the individual proportion of European ancestry increased in the entire cohort, independently of race/color categorization and geographical origin. We concluded that warfarin dosing algorithms that include 'race' terms defined for other populations are clearly not applicable to the heterogeneous and extensively admixed Brazilian population.

Another example was provided by the *CYP3A5* gene, which encodes the enzyme CYP3A5, responsible for the inactivation of several clinically useful drugs, such as the immunosuppressants tacrolimus and cyclosporine. The frequency of the variant *CYP3A5*3* allele (rs776746), which encodes a non-functional CYP3A5 isoform, is less than 10% among sub-Saharan Africans but exceeds 90% among Europeans. However, we have shown that the frequency of *CYP3A5*3* in healthy Brazilians living in Rio de Janeiro, self-identified as White or Black according to the "color/race" categorization of the Brazilian census, was 78% and 32%, respectively, with enormous individual variability within the groups [34]. Thus, the *CYP3A5*3* allele was three times more frequent among self-identified black Brazilians (32%) than black Africans (<10%) and was considerably less common among self-identified white Brazilians (78%) than Europeans (>95%). The African-European admixture of Brazilians provides an explanation for these discrepancies, since irrespective of "color/race" self-identification, most Brazilians share European and African genetic ancestries, and many have also a significant proportion of Amerindian ancestry.

These results show that the heterogeneity of our population cannot be adequately represented by arbitrary "race/color" categories. In a pharmacogenetic context, this implies that each person must be treated as an individual rather than as an "exemplar of a color group" [35].

Based on traditional demographic racial/color methodology, clinical and pharmacological trials in Brazil have usually considered the different regions of the Country as very heterogeneous. Our results show that when viewed under the light of molecular population genetics these classical paradigms are inadequate, since the genomic ancestry of individuals from different geographical regions of Brazil is more uniform than expected.

Our results have considerable sociological relevance for Brazil, because the race question presently figures prominently in Brazilian political life [36]. Among the actions of the State in the sphere of race relations are initiatives aimed at strengthening racial identity, especially "Black identity" encompassing the sum of those self-categorized as Brown or Black in the censuses and government surveys. The argument that non-Whites constitute more than half of the population of the country has been routinely used in arguing for the introduction of public policies favoring the no-White population, especially in the areas of education (racial quotas for entrance to the universities), the labor market, access to land, and so on [36]. Nevertheless, our data presented here do not support such contention, since they show that, for instance, non-White individuals in the North, Northeast and Southeast have predominantly European ancestry and differing proportions of African and Amerindian ancestry.

The relevance of our work also extrapolates the Brazilian borders. Because of its heterogeneous Amerindian, European and African ancestral roots, Brazil has been an important model for the population genetics and pharmacogenetics of admixed populations. Our article demonstrates how critical it is to use genomic tools to reevaluate and modernize previous regional population models established using conventional demographic, anthropological and sociological studies. The same should also be applied to other countries that contain ancestrally admixed populations.

Materials and Methods

Ethics statement

The Research Ethics Committee of the Instituto Nacional do Câncer (INCA) approved in July 15, 2005 the protocol of the study "Characterization of polymorphisms of pharmacogenetic interest and correlation with genetics ancestry" as well as the written Informed Consent form. In August 11, 2008 the Research Ethics Committee of the Instituto Nacional do Câncer (INCA) approved

the enlargement of the study and carried forward the approval of the written consent Informed Consent form. The samples were anonymized after collection.

Populations Studied

We studied 934 unrelated Brazilians from different geographical regions of Brazil (Fig. 1), as described in detail below. Except when noted, the color assignation was obtained by self-assessment in answer to the closed question "What is your color/race?", as done in the Brazilian census by the Instituto Brasileiro de Geografia e Estatística (IBGE). All subjects of this study described themselves as White, Brown or Black (in Portuguese, respectively, "Branco", "Pardo" and "Preto"). These three color categories encompass 99.1% of the Brazilian population. No subjects were self-classified as Indian ("Indígena"), Yellow ("Amarelo") or did not declare a color ("Sem declaração").

The North region was represented by 203 unrelated, healthy individuals (92 men, 111 women) from the Amazonian state of Pará (PA - Fig. 1). The individuals were ascertained in the Municipal Health Clinic in the Sacramenta area of the city of Belém, Pará and self-identified as White (n = 66; 33%), Brown (n = 65; 32%) or Black (n = 72; 35%). They were randomly chosen within each color category.

Two different samples were collected in the Northeast region: (i) 82 individuals were ascertained from healthy students and work personnel at the University of Ceará, in Fortaleza, Ceará (CE - Fig. 1), randomly chosen within the self-classified categories of White (n = 31) or Brown (n = 51) and (ii) 147 healthy individuals were ascertained from the Blood Bank in the city of Ilhéus, Bahia (BA - Fig. 1), randomly chosen within those self-classified as White (n = 48), Brown (n = 26) or Black (n = 73), as described elsewhere [37].

The Southeast sample was made up of 264 unrelated, healthy individuals (162 men, 102 women) from the state of Rio de Janeiro (RJ - Fig. 1), all collected from blood donors, personnel and research students at the Instituto Nacional do Cancer (INCA). The enrolled individuals were self-identified as White (n = 88; 33%), Brown (n = 88; 33%) or Black (n = 88; 33%). They were randomly chosen within each color category. Some of these subjects were analyzed in previous publications [34,38]. The genotype results were very similar with those from a different study with another populations of the city of Rio de Janeiro [38].

Two different samples were obtained in the South region: (i) 189 individuals ascertained from blood donors in Porto Alegre, Rio Grande do Sul (RS - Fig. 1), were not self-identified, but rather had their color assigned by the health professionals who collected their samples, as White (n = 82), Brown (n = 78) or Black (n = 29). They were randomly chosen within each color category. Studies performed in Southern Brazil with large samples have shown that for White interviewers (which was the case in our study) there was no statistical difference between self-classification and interviewer-classification [39] and (ii) 49 self-assessed Black individuals from a Community Center in the city of Joinville, Santa Catarina (SC - Fig. 1).

DNA analysis

DNA from each individual was independently typed for the following 40-biallelic short insertion/deletion polymorphisms (indels): MID-1 (rs3917), MID-15 (rs4181), MID-17 (rs4183), MID-51 (rs16343), MID-89 (rs16381), MID-107 (rs16394), MID-131 (rs16415), MID-132 (rs16416), MID-150 (rs16430), MID-159 (rs16438), MID-170 (rs16448), MID-258 (rs16695), MID-278 (rs16715), MID-420 (rs140709), MID-444 (rs140733), MID-468 (rs140757), MID-470 (rs140759), MID-663 (rs1305047), MID-788 (rs1610874), MID-857 (rs1610942), MID-914 (rs1610997), MID-918 (rs1611001), MID-1002 (rs1611084), MID-1092 (rs2067180), MID-1100 (rs2067188), MID-1129 (rs2067217), MID-1291 (rs2067373), MID-1352 (rs2307548), MID-1428 (rs2307624), MID-1537 (rs2307733), MID-1549 (rs2307745), MID-1586 (rs2307782), MID-1642 (rs2307838), MID-1654 (rs2307850), MID-1759 (rs2307955), MID-1763 (rs2307959), MID-1847 (rs2308043), MID-1861 (rs2308057), MID-1943 (rs2308135), MID-1952 (rs2308144). In this list, The MID number relates to the nomenclature of Weber et al. [40] and the rs numbers relate to dbSNP (http://www.ncbi.nlm.nih.gov/snp/).

This set of 40 indels was previously validated as useful in ancestry estimation through the study of the HGDP-CEPH Diversity Panel [12], which is composed of 1,064 individuals from 52 different worldwide populations distributed in seven geographical regions [11]. The individual results have been deposited in the CEPH Genotype Database (http://www.cephb.fr/en/hgdp/main.php), from which they are available. The multiplex PCR assays and analysis in a MegaBACE 1000 DNA sequencer (GE Healthcare) of the indels were performed exactly as described previously [11].

Data analysis

To estimate the proportion of Amerindian, European and African ancestry in each Brazilian, we applied a model-based clustering algorithm using the *Structure* software version 2.1 [41]. This software uses multilocal genotypes to infer the structure of each population and to allocate probabilistically the proportion of genomic ancestry of individuals in different populations. As parameters we assumed the admixture model, correlated allele frequencies and used 100,000 burn-in steps followed by 900,000 Markov Chain Monte Carlo iterations. We used for reference populations, 158 Europeans, 125 Sub-Saharan Africans and 107 Amerindians of the HGDP-CEPH Diversity Panel, which had been typed as part of our previous studies with the same set of 40 indels [8,11].

Triangular graphs of the genomic proportions of Amerindian, European and African ancestry of each individual were obtained using the Tri-Plot program [42].

For statistical testing of the proportions of European, African and Amerindian ancestry in the different samples we developed a Monte Carlo resampling method, which has the advantage of being completely non-parametric [43], as follows. Since for each individual the relative proportions of European, African and Amerindian ancestries, must necessarily add to unity, a graph with a Cartesian coordinate system, in which the proportion of African ancestry (y-axis) is plotted against the proportion of European ancestry (x-axis) has the same information content as the triangular graph. We can now obtain in a two-dimensional Cartesian graph a point (P_i), whose coordinate values are the mean proportion of European ancestry (X_i; x-axis) and the mean proportion of African ancestry (Y_i; y-axis) for sample i. To compare two samples (say, S_1 and S_2) we can use as a metric the distance between P_1 and P_2 in the two-dimensional graph space, which can be easily calculated, using the Pythagoras' theorem, as $D = [(X1\text{-}X2)^2 + (Y1\text{-}Y2)^2]^{1/2}$, i.e. the Euclidian distance between the means of the two samples. The probability distribution of the distance (D) was established empirically by randomization of the proportion of European ancestry of the two samples and also the proportion of African ancestry of the two samples, respectively, using the software Resampling Stats for Excel 3.2 (Resampling Stats, Inc., Arlington, Va., USA). The probability of random occurrence of a given value of the distance D was then established after 10,000 cycles. The significance level (two-tailed) was *a priori* established at $\alpha = 0.05$.

Supporting Information

Table S1 Frequencies of the short allele of the 40 short insertion-deletion polymorphic loci used, in four regions of Brazil.
(DOC)

Table S2 To estimate the significance of the pairwise differences observed between the samples of the diverse regions we used a specially designed Monte Carlo randomization test of the Euclidian distance D between the means of the European and African ancestries. In the table, the observed distances are in the cells above the diagonal and the probability of obtaining the observed distances by chance is shown in the cells below the diagonal. The cells in bold italic type are significant, even after Bonferroni's correction ($P<0.008$).
(DOC)

Author Contributions

Conceived and designed the experiments: SDJP GSK. Performed the experiments: MFM JPG FSGK FK LAVM MRM JAP CR VAS. Analyzed the data: SDJP GSK MHH AKCRS EBO MARS. Contributed reagents/materials/analysis tools: GDP RCM MOM MEAM FRS. Wrote the manuscript: SDJP GSK.

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Influence of Genomic Ancestry on the Distribution of *SLCO1B1*, *SLCO1B3* and *ABCB1* Gene Polymorphisms among Brazilians

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Abstract: The frequency distribution of SNPs and haplotypes in the *ABCB1*, *SLCO1B1* and *SLCO1B3* genes varies largely among continental populations. This variation can lead to biases in pharmacogenetic studies conducted in admixed populations such as those from Brazil and other Latin American countries. The aim of this study was to evaluate the influence of self-reported colour, geographical origin and genomic ancestry on distributions of the *ABCB1*, *SLCO1B1* and *SLCO1B3* polymorphisms and derived haplotypes in admixed Brazilian populations. A total of 1039 healthy adults from the north, north-east, south-east and south of Brazil were recruited for this investigation. The c.388A>G (rs2306283), c.463C>A (rs11045819) and c.521T>C (rs4149056) SNPs in the *SLCO1B1* gene and c.334T>G (rs4149117) and c.699G>A (rs7311358) SNPs in the *SLCO1B3* gene were determined by Taqman 5′-nuclease assays. The *ABCB1* c.1236C>T (rs1128503), c.2677G>T/A (rs2032582) and c.3435C>T (rs1045642) polymorphisms were genotyped using a previously described single-base extension/termination method. The results showed that genotype and haplotype distributions are highly variable among populations of the same self-reported colour and geographical region. However, genomic ancestry showed that these associations are better explained by a continuous variable. The influence of ancestry on the distribution of alleles and haplotype frequencies was more evident in variants with large differences in allele frequencies between European and African populations. Design and interpretation of pharmacogenetic studies using these transporter genes should include genomic controls to avoid spurious conclusions based on improper matching of study cohorts from Brazilian populations and other highly admixed populations.

The efficacy of drug therapy results from complex interplay between multiple processes that govern drug disposition and response. Although passive diffusion accounts for cellular uptake of some drugs and metabolites, increased emphasis is being placed on the role of membrane transporters in absorption of oral medications across the gastrointestinal tract. Many current United States Food and Drug Administration (FDA)-approved drugs are substrates of these transporters [1].

The MDR1 multidrug transporter is one of the better-characterized members of the ATP-binding cassette (ABC) family of transporters. Although this transporter was initially identified in the context of multidrug resistance (MDR) against anticancer drugs, its range of known drugs and substrates has greatly expanded. In addition to chemotherapeutic drugs, the MDR1 transporter has been found to transport a wide variety of substrates representing nearly every category of clinically important drugs, including antiarrhythmics, antidepressants, antipsychotics and antivirals [2–4].

More than 50 polymorphisms have been described in the *ABCB1* gene [5]. Single-nucleotide polymorphisms (SNPs) c.1236C>T (Gly411Gly) in exon 12, c.2677G>T/A (Ala893-Ser/Thr) in exon 21 and c.3435C>T (Ile1145Ile) in exon 26 are three SNPS that have been most commonly investigated in pharmacogenetic studies [6,7]. The c.2677G>T/A SNP causes an amino acid change within a structurally important transmembrane domain of the translated protein. However, the effects of this polymorphism are controversial and drug-specific [6,8–11]. The c.3435C>T SNP is associated with a decrease in mRNA stability and lower expression levels [12]. The organic anion-transporting polypeptides (OATPs) are sodium-independent transporters encoded by genes of the solute carrier family *SLCO*. These transporters are present in the basolateral membrane of hepatocytes and are major determinants of uptake of several drugs from the portal circulation into hepatocytes [1,13,14]. OATP1B1 and OATP1B3 are encoded by *SLCO1B1* and *SLCO1B3*, respectively. Several SNPs and other sequence variations have been described

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in *SLCO1B1* [15,16], some of which are associated with altered transport *in vivo* and *in vitro*. The c.388A>G (Asn130Asp), c.463C>A (Pro155Thr) and c.521T>C (Val174Ala) SNPs and their derived haplotypes were the focus of several investigations into drug transport, efficacy and tolerance. Genetic variants of the *SLCO1B1* gene have been shown to reduce hepatic uptake and increase plasma concentrations of statins [17]. *In vivo* studies suggested that the c.388A>G variant is associated with increased OATP1B1 activity, and the c.521T>C SNP is associated with reduced transport *in vitro*. A genome-wide study from the SEARCH collaborative group [18] showed that carriers of 521C allele taking 80 mg of simvastatin were found to be at a significantly increased risk of myopathy (odds ratio 4.5; 95% confidence interval 2.6–7.7).

The most common SNPs at the *SLCO1B3* locus are c.699G>A (Met233Ile) and c.344T>G (Ser112Ala). These SNPs were associated with altered transport activity *in vitro* in COS-7 cells [19] but not in other cell lines [20,21]. The influence of the 699G>A/334T>G haplotype in digoxin pharmacokinetics was recently demonstrated by Tsujimoto *et al.* [22].

The Brazilian population was formed by successive migratory waves. Amerindian people occupied the Brazilian territory when the Portuguese arrived in 1500 and colonized the country. Then, between the 16th and 19th centuries, Africans were brought to Brazil as slaves. In addition to the Portuguese, other migratory waves occurred in the 19th and 20th centuries, mainly from Italy, Germany and Spain [23]. All of these migratory events contributed to the formation of a multi-ethnic and highly admixed population. This heterogeneity was documented in several genetic studies that used either uniparental or autosomal markers to demonstrate a typical, although non-uniform, tri-ethnic (European, African and Amerindian) pattern for the Brazilian population. This admixture process occurred in different ways in the various geographical regions of the country. In north-eastern Brazil, the African contribution is high; in the north, the contribution of Native Americans is pronounced; and in the south, there are reduced Amerindian and African influences when compared with the other geographical regions [23–27].

The frequency distribution of SNPs and haplotypes in the *ABCB1*, *SLCO1B1* and *SLCO1B3* genes varies largely among continental populations [17,28–32]. This variation can lead to biases in pharmacogenetic studies conducted in admixed populations such as those from Brazil and other Latin American countries. Because the proportion of Amerindian, African and European ancestry varies in each individual and between Brazilian regions, the 'racial' classification officially used in Brazil based on self-perceived skin colour [33,34] can misrepresent real stratifications in pharmacogenetic association studies. In the present study, we evaluated the influence of 'racial' classifications, geographical origins and genetic ancestry in the distribution of *ABCB1*, *SLCO1B1* and *SLCO1B3* polymorphisms and haplotypes in a representative sample of the Brazilian population.

Subjects and Methods

Study population. The study cohort consisted of 1039 healthy adults recruited from the north, north-east, south and south-east regions of Brazil. Sample collection and procedures for individual ancestry determination were performed as described previously [34]. Briefly, each individual was asked to self-identify according to the classification scheme adopted by the official Brazilian Census [33], which relies on self-perception of skin colour. From this self-identification, the subjects were distributed into the following three groups: white (*branco*, n = 342), brown (*pardo*, n = 352) and black (*preto*, n = 345). All enrolled subjects provided their informed consent to participate. The study protocol was approved by the ethics committees of the institutions that participated in blood sample collection. Of the 1039 participants, 934 (89%) were genotyped for a set of 40 biallelic short insertion/deletion polymorphisms (indels) previously validated as informative markers for ancestry [35,36]. With these data, the individual proportions of European, African and Amerindian genetic ancestry were estimated using Structure version 2.1 [37].

Genotyping. Genomic DNA was isolated from peripheral blood by standard procedures. The *SLCO1B1* 388A>G (rs2306283), 463C>A (rs11045819) and 521T>C (rs4149056) SNPs and the *SLCO1B3* 334T>G (rs4149117) and 699G>A (rs7311358) SNPs were determined by allelic discrimination with Taqman 5′-nuclease assays according to the manufacturer's recommended protocols. The *ABCB1* 1236C>T (rs1128503), c.2677G>T/A (rs2032582) and c.3435C>T (rs1045642) polymorphisms were genotyped using a previously described [31] single-base extension/termination method on the SNaPshot multiplex system from Applied Biosystems (Foster City, CA, USA).

Statistical analyses. Allele and genotype frequencies were estimated by gene counting. Deviation from Hardy–Weinberg equilibrium was assessed through chi-square tests. Haplotype frequencies and linkage disequilibrium were estimated using the Multiple Locus Haplotype Analysis program (version 3.0) [38].

Statistical associations between allele, genotype and haplotype distributions, and self-reported colour or geographical region were inferred by fitting multinomial log-linear models. This procedure obviates the need for correction for multiple comparisons because the main effects and interaction terms are tested simultaneously within each regression context. Pearson correlation and Kruskal–Wallis one-way ANOVA were performed to examine the association between the *ABCB1*, *SLCO1B1* and *SLCO1B3* polymorphisms and the genetic ancestry of the population. Statistical analysis was performed using the SPSS18.0 statistical package for Windows® (Albany, NY, USA) and Graph Pad Prism for Windows® (La Jolla, CA, USA). A p-value < 0.05 was considered significant in all analyses.

Results

Distribution of ABCB1, SLCO1B1 and SLCO1B3 polymorphisms among Brazilians according to self-categorization and geographical region.

Allele and genotype distributions of *ABCB1*, *SLCO1B1* and *SLCO1B3* SNPs among Brazilians stratified by geographical region and self-reported colour are presented in table 1, and their derived haplotypes are presented in table 2. The genotype frequencies observed for all studied polymorphisms did not reveal statistically significant differences compared to those expected under Hardy–Weinberg equilibrium.

All *ABCB1* SNPs were in linkage disequilibrium. The c.1236T allele was in linkage disequilibrium with c.2677G and c.3435C alleles (D' = 0.886 and D' = 0.753; $p < 0.001$).

Table 1.

Allele and genotype frequency of *ABCB1* and *SLCO1B1* polymorphisms according to self-reported colour and geographical region.

		Geographical region														
		Brazil			North			North-east			South-east			South		
SNPs		White (n = 342)	Brown (n = 352)	Black (n = 345)	White (n = 78)	Brown (n = 88)	Black (n = 88)	White (n = 88)	Brown (n = 88)	Black (n = 87)	White (n = 88)	Brown (n = 88)	Black (n = 88)	White (n = 88)	Brown (n = 88)	Black (n = 82)
ABCB1																
1236C>T	CC	34.0	41.2	50.1	29.9	35.7	37.5	37.2	33.3	47.1	37.9	45.5	49.4	30.7	50.0	67.9
	CT	46.2	47.3	38.5	41.6	48.8	50.0	48.8	50.6	41.4	44.8	48.9	39.1	48.9	40.9	22.2
	TT	19.8	11.5	11.4	28.6	15.5	12.5	14.0	16.1	11.5	17.2	5.7	11.5	20.5	9.1	9.9
	C	57.1	64.8	69.4	61.6	60.1	62.5	61.6	58.6	67.8	60.3	69.9	69.0	55.1	70.5	79.0
	T	42.9	35.2	30.6	38.4	39.9	37.5	38.4	41.4	32.2	39.7	30.1	31.0	44.9	29.5	21.0
2677G>T/A	GG	33.1	45.8	60.9	35.6	36.9	47.7	36.0	31.0	54.0	34.5	51.7	67.1	26.7	66.2	76.6
	G/NonG	49.8	44.0	31.6	41.1	47.6	37.2	52.3	55.2	37.9	48.8	42.5	30.6	55.9	28.4	19.5
	NonG/NonG	17.0	10.2	7.5	23.3	15.5	15.1	11.6	13.8	8.0	16.7	5.7	2.4	17.4	5.4	3.9
	G	58.1	67.8	76.7	56.2	60.7	66.3	62.2	58.6	73.0	58.9	73.0	82.4	54.7	80.4	86.4
	NonG	41.9	32.2	23.3	43.8	39.3	33.7	37.8	41.4	27.0	41.1	27.0	17.6	45.3	19.6	13.6
3435C>T	CC	29.3	36.5	46.8	28.0	27.6	37.5	33.3	25.0	44.2	29.9	41.4	50.6	25.6	52.3	55.6
	CT	48.7	48.0	41.8	45.3	50.6	48.9	49.4	53.4	41.9	50.6	50.6	41.2	48.8	37.2	34.6
	TT	22.1	15.5	11.5	26.7	21.8	13.6	17.2	21.6	14.0	19.5	8.0	8.2	25.6	10.5	9.9
	C	53.6	60.5	67.6	50.7	52.9	61.9	58.0	51.7	65.1	55.2	66.7	71.2	50.0	70.9	72.8
	T	46.4	39.5	32.4	49.3	47.1	38.1	42.0	48.3	34.9	44.8	33.3	28.8	50.0	29.1	27.2
SLCO1B1																
388A>G	AA	24.9	17.6	12.2	26.9	26.1	19.3	23.9	19.3	14.9	25.0	10.2	8.0	23.9	14.8	6.1
	AG	51.2	46.0	45.8	52.6	50.0	39.8	46.6	51.1	56.3	51.1	42.0	50.0	54.5	40.9	36.6
	GG	24.0	36.4	42.0	20.5	23.9	40.9	29.5	29.5	28.7	23.9	47.7	42.0	21.6	44.3	57.3
	A	50.4	40.6	35.1	53.2	51.1	39.2	47.2	44.9	43.1	50.6	31.3	33.0	51.1	35.2	24.4
	G	49.6	59.4	64.9	46.8	48.9	60.8	52.8	55.1	56.9	49.4	68.8	67.0	48.9	64.8	75.6
463C>A	CC	79.5	79.3	84.1	83.3	84.1	87.5	80.7	72.7	77.0	78.4	78.4	81.8	76.1	81.8	90.2
	CA	19.3	19.3	15.4	16.7	15.9	12.5	18.2	26.1	21.8	18.2	19.3	18.2	23.9	15.9	8.5
	AA	1.2	1.4	0.6	0	0	0	1.1	1.1	1.1	3.4	2.3	0	0	2.3	1.2
	C	89.2	88.9	91.7	89.8	92.0	93.8	89.8	85.8	87.9	87.5	88.1	90.9	88.1	89.8	94.5
	A	10.8	11.1	8.3	10.2	8.0	6.3	10.2	14.2	12.1	12.5	11.9	9.1	11.9	10.2	5.5
521T>C	TT	76.0	74.4	80.9	78.2	69.3	78.4	77.3	75.0	78.2	72.7	70.5	86.4	76.1	83.0	80.5
	TC	22.8	23.6	19.1	21.8	27.3	21.6	21.6	23.9	21.8	70.5	28.4	13.6	22.7	14.8	19.5
	CC	1.2	2.0	0	0	3.4	0	1.1	1.1	0	86.4	1.1	0	1.1	2.3	0
	T	87.4	86.2	90.4	89.1	83.0	89.2	88.1	86.9	89.1	85.2	84.7	93.2	87.5	90.3	90.2
	C	12.6	13.8	9.6	10.9	17.06	10.8	11.9	13.1	10.9	14.8	15.3	6.8	12.5	9.7	9.8

Data expressed as percentage.

Table 2.
Multinomial log-linear analyses of *ABCB1*, *SLCO1B1* and *SLCO1B3* polymorphisms among Brazilians according to self-reported colour and geographical region.

SNPs		Colour	Geographical region	Colour/ geographical region
ABCB1				
1236C>T	Alleles	<0.001	0.003	0.066
	Genotypes	<0.001	0.011	0.092
2677G>T/A	Alleles	<0.001	<0.001	<0.001
	Genotypes	<0.001	<0.001	0.004
3435C>T	Alleles	<0.001	0.003	0.016
	Genotypes	<0.001	0.008	0.142
	Haplotypes	<0.001	0.001	0.013
SLCO1B1				
388A>G	Alleles	<0.001	<0.001	0.012
	Genotypes	<0.001	0.003	0.034
463C>A	Alleles	0.152	0.058	0.656
	Genotypes	0.411	0.059	0.376
521T>C	Alleles	0.039	0.677	0.347
	Genotypes	0.014	0.898	0.537
	Haplotypes	<0.001	0.001	0.003
SLCO1B3				
334T>G/699G>A	Haplotypes	<0.001	0.004	0.033

The c.2677G allele is also in linkage disequilibrium with the c.3435C allele (D' = 0.872 $p < 0.001$).

Eight haplotypes were derived from *ABCB1* SNPs, but only three (C-G-C, C-G-T and T-NonG-T) occurred with frequencies >5% and together accounted for 90% of the diversity observed in the study population; therefore, only these three haplotypes were included in the analyses.

The *SLCO1B1* SNPs were in linkage disequilibrium. The c.463A allele showed complete linkage disequilibrium with c.388G and c.521T (D' = 1.000 and $p < 0.001$). The c.388G allele was in linkage disequilibrium with the c.521T allele (D' = 0.886 $p < 0.001$). Therefore, five different *SLCO1B1* haplotypes were observed: **1a*, **1b*, **5*, **14* and **15*.

SLCO1B3 gene polymorphisms were in complete linkage disequilibrium, forming c.334T/c.699G and c.334G/c.699A haplotypes (D' = 1.000 and $p < 0.001$). No individual SNP analyses were performed with *SLCO1B3* because of complete linkage disequilibrium.

The multinomial log-linear analyses showed highly significant associations between *ABCB1* SNPs frequencies, colour and geographical region (table 3). These results reflect the trends for decreasing frequency of c.1236T, c.2677NonG and c.3435T alleles from white to black individuals in the north, south-east and southern regions. The geographical region association is explained by differences in frequency distribution of these alleles in self-reported colour from north to the south region. The interaction of region with colour was significantly associated with the c.2677G>T/A polymorphism (table 3). *SLCO1B1* c.388A>G and c.521T>C polymorphisms were associated with colour in the multinomial log-linear analyses (table 3). The c.388A>G association reflects the increasing frequency of c.388G variant from black to white in the north, north-east and southern regions, and the c.521T>C association reflects the increasing frequency of c.521C variant from white to black in the south-east and south regions. The c.388A>G was also associated with geographical region and with the interaction between region and colour (table 3).

ABCB1, *SLCO1B1* and *SLCO1B3* haplotypes among Brazilians stratified by geographical region and self-reported colour are shown in table 4. The multinomial log-linear analyses revealed significant effects of self-reported colour, geographical region and the interaction between colour and region on *ABCB1*, *SLCO1B1* and *SLCO1B3* haplotypes distribution among Brazilians (table 3).

Association of ABCB1, SLCO1B1 and SLCO1B3 polymorphisms and genomic ancestry.
Genomic ancestry based on the individual proportions of European, African and Amerindian ancestry independent of self-reported colour was investigated in this cohort as a continuous variable. Significant correlations between European ancestry and *ABCB1* c.1236T ($p = 0.0031$), *ABCB1* c.2677NonG ($p = 0.0013$) and *ABCB1* c.3435T ($p = 0.0003$) alleles, and correlation of *SLCO1B1* 388G with African ancestry ($p = 0.0005$) were found. No correlation was observed for *SLCO1B1* c.463A and c.521C alleles ($p = 0.22$; $p = 0.12$, respectively). As expected from the individual SNP analyses, *ABCB1* T-NonG-T haplotype was significantly correlated with European ancestry ($p = 0.0008$), whereas *SLCO1B1*1b* frequency increases with African ancestry ($p = 0.0012$). The *SLCO1B3 G-A* ($p = 0.0003$) haplotype was also correlated with European ancestry ($p = 0.0003$). The average proportions of Amerindian, African and European ancestry were compared between *ABCB1*, *SLCO1B1* and *SLCO1B3* genotypes and haplotypes (tables 5 and 6). The T-NonG-T haplotype is associated with a lower proportion of African ancestry and a higher proportion of

Table 3.
Frequency of SNP haplotypes from *ABCB1*, *SLCO1B1* and *SLCO1B3* genes found in the Brazilian population.

Gene	SNP				
ABCB1	1236T>C	2677G>T/A	3435C>T	Haplotypes	Frequency
	C	G	C	C-G-C	54.8 (53.2; 56.8)
	C	G	T	C-G-T	7.1 (6.1; 8.0)
	C	NonG	C	C-NonG-C	1.1 (0.7; 1.4)
	C	NonG	T	C-NonG-T	1.2 (0.8; 1.5)
	T	G	C	T-G-C	3.7 (3.0; 4.3)
	T	G	T	T-G-T	2.2 (1.6; 2.7)
	T	NonG	C	T-NonG-C	1.2 (0.8; 1.5)
	T	NonG	T	T-NonG-T	28.7 (27.3; 30.6)
SLCO1B1	388A>G	463C>A	521T>C		
	A	C	T	SLCO1B1*1a	41.6 (40.2; 43.7)
	G	C	T	SLCO1B1*1b	36.5 (34.2; 37.7)
	A	C	C	SLCO1B1*5	0.4 (0.6; 1.3)
	G	A	T	SLCO1B1*14	11.5 (9.8; 12.1)
	G	C	C	SLCO1B1*15	10.0 (8.9; 11.0)
SLCO1B3	334T>G	699G>A			
	T	G		T-G	29.8 (28.3; 31.6)
	G	A		G-A	70.2 (68.3; 71.5)

Frequency expressed as percentage (CI 95%).

European ancestry in the study population (table 6). At the *SLCO1B1* locus, the haplotype **1b* demonstrates statistically significant differences in genetic ancestry proportions compared to other haplotypes. This haplotype has only the c.388G variant and is strongly associated with a higher proportion of African ancestry (table 6). The SLCO1B3 T-G haplotype was associated with a higher proportion of African ancestry, whereas its counterpart, the G-A haplotype, was associated with a higher proportion of European ancestry (table 6).

Discussion

The present investigation reports the first large-scale study of *ABCB1*, *SLCO1B1* and *SLCO1B3* polymorphism diversity in a representative sample of the Brazilian population. Within a country the size of Brazil, population composition varies widely among regions. Therefore, it is not unexpected that population heterogeneity and diversity influenced the distribution of *ABCB1*, *SLCO1B1* and *SLCO1B3* genotypes and haplotypes within self-reported colour groups, across geographical regions, and according to the proportions of European, Amerindian and African genetic ancestry [23,34,39].

Individuals differ genetically in their susceptibility to particular diseases and their responses to various drugs. However, personalized treatments are difficult to develop because disease susceptibility and drug response generally have poorly characterized genetic determinants. It is therefore tempting to use the ethnicity of patients as a proxy for some of the variation in allele frequencies in genes that could underlie these traits [40]. Transporter gene polymorphisms are known to occur at variable frequencies in different continental populations [14,17,29,30,32,40], but fine geographical distribution information is not available for most populations.

ABCB1 polymorphisms display a marked interethnic variation that is possibly a result of selection owing to specific environmental pressures. The observed trend in the Brazilian population for increased frequency of the *ABCB1* c.1236T, c.2677nonG and c.3435T alleles and the T-NonG-T haplotype as the average proportion of European ancestry increases is compatible with the reported frequencies of these variants in Europe and sub-Saharan Africa [28,31,32,41–45]. The allele and haplotype distributions are similar to those reported in other Brazilian studies, but they differ from those described in African American or European-derived American populations. This is probably due to different levels of admixture in these populations [31,32,45]. These results did not differ when self-reported colour or genomic ancestry was considered.

The *SLCO1B1* c.388A>G SNP was significantly associated with colour and geographical region and the interaction between these factors. Our study demonstrates that the *SLCO1B1* c.388G variant was also strongly associated with decreases in European ancestry and increases in African ancestry. The c.521T>C SNP distribution was weakly associated with colour; however, this polymorphism was not associated with genetic ancestry. The c.463C>A SNP was not associated with colour, region or genetic ancestry. The haplotype frequencies of *SLCO1B1* varied among regions and self-reported skin colour, but when genomic ancestry was considered, only the *SCLO1B1*1b* haplotype was associated with increased African ancestry. The global analyses reported by Pasanen *et al.* [30] showed that the c.521T>C SNP varied markedly between populations. The lowest

Table 4.
Haplotype frequency of *ABCB1*, *SLCO1B1* and *SLCO1B3* haplotypes according to self-reported colour and geographical region.

	Geographical region											
	Brazil			North			North-east			South-east			South		
Haplotypes	White (n = 342)	Brown (n = 352)	Black (n = 345)	White (n = 78)	Brown (n = 88)	Black (n = 88)	White (n = 88)	Brown (n = 88)	Black (n = 87)	White (n = 88)	Brown (n = 88)	Black (n = 88)	White (n = 88)	Brown (n = 88)	Black (n = 82)
ABCB1															
C-G-C	47.9	55.4	60.9	54.5	47.2	62.6	42.8	49.4	52.8	50.0	61.4	60.9	43.7	64.0	67.9
C-G-T	7.2	7.2	7.0	5.7	8.5	5.2	7.2	8.6	4.5	8.0	6.3	6.3	8.0	5.2	12.3
T-NonG-T	37.7	28.7	20.0	34.1	36.9	25.3	40.8	35.1	28.4	35.6	24.4	14.9	40.8	18.0	10.5
Others	7.1	8.7	12.1	5.7	7.4	6.9	9.2	6.9	14.2	6.3	8.0	17.8	7.5	12.8	9.3
SLCO1B1															
SLCO1B1*1a	49.7	40.2	35.1	51.3	50.6	39.2	46.6	44.9	43.1	50.0	30.7	33.0	51.1	34.7	24.4
SLCO1B1*1b	27.2	34.9	47.2	29.5	25.0	44.3	32.4	27.3	33.9	22.7	42.0	51.1	24.4	45.5	60.4
SLCO1B1*5	0.7	0.3	0	1.9	0.6	0	0.6	0	0	0.6	0	0	0	0.6	0
SLCO1B1*14	10.5	11.2	8.3	8.3	8.0	6.3	9.1	14.8	12.1	12.5	11.9	9.1	11.9	10.2	5.5
SLCO1B1*15	11.8	13.4	9.4	9.0	15.9	10.2	11.4	13.1	10.9	14.2	15.3	6.8	12.5	9.1	9.8
SLCO1B3															
T-G	20.5	30.7	38.0	19.9	25.0	27.8	25.0	25.0	37.9	19.5	35.8	45.3	17.6	36.9	41.5
G-A	79.5	69.3	62.0	80.1	75.0	72.2	75.0	75.0	62.1	80.5	64.29	54.7	82.4	63.1	58.5

Data expressed as percent.

c.521C frequencies were observed in sub-Saharan Africans and the highest in Native American populations. In contrast, the c.388G allele at the c.388A>G SNP had a higher prevalence in Africa. Pasanen *et al.* [30] have also shown that the *SLCO1B1*1b* haplotype was the most common haplotype in African and Native American populations, whereas haplotype **5* was only seen in Europe and the Middle East. In our admixed population, all haplotypes were identified in individuals self-classified as white or brown. However, not all haplotypes were identified in those self-classified as black, despite high levels of admixture [36]. Although haplotype **1b* occurs with a high frequency in Native Americans, we did not observe a significantly higher contribution of Amerindian ancestry in carriers of this haplotype in admixed Brazilians.

The *SLCO1B3* gene has not been previously investigated to the extent of *SCLO1B1* and *ABCB1*. This distinguishes between what has been previously described in this paper and the previous investigations into other groups. The frequencies of the SLCO1B3 c.344T>G and c.699G>A SNPs are similar in Caucasian and Asian populations but markedly lower in sub-Saharan Africans. The frequencies of the SLCO1B3 c.344T>G and c.699G>A SNPs were the major alleles in both Caucasian and Asian populations, whereas the major alleles in sub-Saharan Africans were c.344T and c.699G [21]. In Brazilians, the *SLCO1B3* haplotypes were associated with self-reported colour, geographical region, the interaction between colour and geographical region and genomic ancestry. The G-A haplotype was strongly associated with European ancestry, which was expected from worldwide population studies.

Geographical, social and cultural barriers have given rise to reproductively isolated human populations. Within these populations, random drift and/or natural selection has produced genetic differentiation. Historically, proxies such as skin colour, race and ethnicity have been used to make inferences regarding population structure, even in the absence of corroborative genetic data [46]. As a result, there is a large body of literature comparing phenotypes between cohorts defined, for example, as 'blacks' and 'whites'. It has also been shown that even when using proxies such as skin colour to match cases and controls, hidden admixture can still occur. This has been demonstrated by Shriver *et al.* [47] in European American populations and by the present study in Brazilian populations.

One significant consequence of population genetic structures is increased confounding in case–control association studies. In areas where people from different regions have mixed extensively, such as in Brazil, the connection between skin colour and ancestry has been substantially weakened [39].

One major goal of the present study was to quantify the correspondence between self-identified race/ethnicity and genetic ancestry in genes that are important for pharmacogenetic studies. In addition, because case and control subjects are sometimes recruited from different geographical regions and because pooled samples are sometimes used for

Table 5.
Genetic ancestry proportion according *ABCB1* and *SCLO1B1* genotypes.

SNP	Genotypes	Amerindian	African	European
ABCB1				
1236C>T	CC	0.110 (0.10; 0.12)	0.305 (0.28; 0.33)	0.585 (0.55; 0.62)
	CT	0.117 (0.10; 0.13)	0.235 (0.21; 0.26)	0.648 (0.62; 0.68
	TT	0.122 (0.10; 0.15)	0.194 (0.15; 0.23)	0.684 (0.63; 0.74)
p		0.73	<0.001	0.001
2677G>T/A	GG	0.113 (0.10; 0.13)	0.328 (0.30; 0.36)	0.559 (0.53; 0.59)
	G/NonG	0.114 (0.10; 0.13)	0.203 (0.18; 0.23)	0.683 (0.65; 0.71)
	NonG/NonG	0.127 (0.10; 0.16)	0.153 (0.12; 0.19)	0.721 (0.67; 0.77)
p		0.44	<0.001	<0.001
3435C>T	CC	0.112 (0.10; 0.13)	0.321 (0.29; 0.35)	0.568 (0.53; 0.60)
	CT	0.113 (0.10; 0.13)	0.242 (0.22; 0.27)	0.645 (0.62; 0.67)
	TT	0.128 (0.10; 0.16)	0.162 (0.13; 0.19)	0.710 (0.67; 0.75)
p		0.86	<0.001	<0.001
SLCO1B1				
388A>G	AA	0.124 (0.10; 0.15)	0.174 (0.14; 0.21)	0.702 (0.66; 0.74)
	AG	0.118 (0.10; 0.13)	0.244 (0.22; 0.27)	0.638 (0.61; 0.67)
	GG	0.106 (0.09; 0.12)	0.321 (0.29; 0.35)	0.572 (0.54; 0.61)
p		0.36	<0.001	<0.001
463C>A	CC	0.121 (0.11; 0.13)	0.265 (0.25; 0.29)	0.614 (0.59; 0.64)
	CA	0.089 (0.07; 0.11)	0.232 (0.19; 0.27)	0.679 (0.64; 0.72)
	AA	0.112 (0.03; 0.19)	0.138 (0.03; 0.25)	0.750 (0.56; 0.94)
p		0.18	0.14	0.02
521T>C	TT	0.109 (0.10; 0.12)	0.270 (0.25; 0.29)	0.621 (0.60; 0.64)
	TC	0.132 (0.11; 0.15)	0.216 (0.18; 0.25)	0.652 (0.61; 0.69)
	CC	0.173 (−0.01; 0.36)	0.226 (0.22; 0.39)	0.601 (0.37; 0.83)
p		0.32	0.16	0.56

Genetic ancestry is expressed as mean (CI 95%).

Table 6.
Genetic ancestry proportion according *ABCB1*, *SCLO1B1* and *SLCO1B3* haplotypes.

Haplotypes	Amerindian	African	European
ABCB1			
C-G-C	0.111 (0.10; 0.12)	0.290 (0.27; 0.31)	0.599 (0.58; 0.62)
C-G-T	0.123 (0.10; 0.15)	0.227 (0.18; 0.27)	0.650 (0.60; 0.70)
T-NonG-T	0.120 (0.11; 0.13)	0.183 (0.16; 0.20)	0.698 (0.67; 0.72)
p	0.95	<0.001	<0.001
SLCO1B1			
SLCO1B1*1a	0.119 (0.11; 0.13)	0.215 (0.20; 0.23)	0.665 (0.65; 0.69)
SLCO1B1*1b	0.110 (0; 0.52)	0.329 (0.01; 0.14)	0.322 (0.38; 0.95)
SLCO1B1*5	0.261 (0.10; 0.12)	0.075 (0.31; 0.35)	0.663 (0.54; 0.59)
SLCO1B1*14	0.092 (0.11; 0.15)	0.223 (0.19; 0.25)	0.685 (0.61; 0.69)
SLCO1B1*15	0.132 (0.08; 0.11)	0.222 (0.19; 0.26)	0.647 (0.64; 0.73)
p	0.11	<0.001	<0.001
SLCO1B3			
T-G	0.107 (0.10; 0.12)	0.337 (0.31; 0.36)	0.557 (0.53; 0.58)
G-A	0.119 (0.11; 0.13)	0.225 (0.21; 0.24)	0.657 (0.64; 0.67)
p	0.80	<0.001	<0.001

Genetic ancestry is expressed as mean (CI 95%).

genome-wide association studies' (GWAS) purposes, it is important to evaluate the assumption that matching only at the level of self-identified race/ethnicity is sufficient. In the present investigation, we demonstrate that ancestry in Brazilians is better explained by a continuous ancestral variable. This variation could lead to stratification bias if genomic controls are not included in pharmacogenetic analyses. Moreover, the intrinsic heterogeneity of the Brazilian population must be acknowledged in the design and interpretation of pharmacogenetic studies using these transporter genes to avoid spurious conclusions based on improper matching of study cohorts.

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SLCO1B1 gene variability influences lipid-lowering efficacy on simvastatin therapy in Southern Brazilians

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Abstract

Background: Variants in uptake and efflux transporters can influence diverse statin pharmacokinetics in different populations. This study aimed to investigate the influence of *SLCO1B1* gene polymorphism on simvastatin treatment efficacy in a Brazilian population of European ancestry.
Methods: Two hundred and sixteen hypercholesterolemic patients were treated with 20 mg/day simvastatin for 6 months. Plasma lipid and lipoprotein levels were measured at baseline and after 2 and 6 months of treatment. The single nucleotide polymorphisms (SNPs) c.388A>G, c.463C>A and c.521T>C at *SLCO1B1* gene were determined by allelic discrimination with TaqMan 5′-nuclease assays. The 388G allele was observed in 160 patients, the 521 C allele was observed in 64 individuals, whereas 61 subjects were 463 A allele carriers.
Results: Carriers of the *SLCO1B1* 388G allele had a greater reduction of total cholesterol and LDL cholesterol with simvastatin treatment, when compared with 56 388A homozygotes (–28.8% vs. –15.8%, p=0.005 and –39.0% vs. –30.6%, p=0.003; respectively). The c.463C>A and c.521T>C SNPs were not associated with simvastatin treatment. The *SLCO1B1* haplotypes showed no statistically significant differences in mean percentage reductions in lipid and lipoprotein levels after simvastatin treatment.
Conclusions: The present study suggests that the *SLCO1B1* c.388A>G polymorphism could play a role in the inter-individual variation of clinical response to simvastatin in Brazilians. These results add to those that suggest that the effects of *SLCO1B1* variants may be statin specific.

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Introduction

Cardiovascular disease is the most common cause of death in the world, with evidence of high low-density lipoprotein-cholesterol (LDL-C) and low high-density lipoprotein-cholesterol (HDL-C) concentrations as important independent risk factors (1). Cholesterol-lowering therapy is the central approach in the primary and secondary prevention of cardiovascular disease. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are the most prescribed drugs for the treatment of dyslipidemia and prevention of cardiovascular disease. Statins decrease cholesterol synthesis by competitively inhibiting HMG-CoA reductase, the enzyme responsible for catalyzing the conversion of HMG-CoA to mevalonate, a precursor of cholesterol (2). Inhibition of the HMG-CoA reductase in the liver increases the expression of LDL receptors in the hepatocyte plasma membrane, enhancing the removal of LDL particles from blood and reducing plasma total and LDL-C concentrations (3). In addition to its role as the target organ, the liver plays an essential role in drug clearance from the circulation and elimination through metabolic processes catalyzed by several cytochrome P450 isozymes and phase two enzyme reactions, sufficiently polarized metabolites are then excreted by efflux carriers expressed at either the apical or canalicular membrane (4, 5). There is considerable variation in inter-individual response to statin therapy, but the origins of this variation are still poorly understood. It is explained, in part, by genetic factors that affect drug pharmacodynamics and/or pharmacokinetics (4, 6).

The organic anion-transporting polypeptides (OATPs) are sodium independent transporters encoded by genes of the solute carriers family *SLCO*. The mechanism of its substrate transport is not completely understood, although it has been suggested that OATPs translocate their substrates through a central, positively charged pore in a so-called rocker-switch type of mechanism (7, 8). The transport is thought to be electroneutral and is independent of sodium, chloride and potassium gradients; membrane potential; and ATP levels (8, 9). These transporters are present in the basolateral membrane of hepatocytes, in addition to OATP1B1, two other OATPs, OATP1B3 and OATP2B1, are also highly expressed in human liver (8, 10). OATP1B1 is known to transport a number of endogenous and exogenous substances, including atorvastatin, cerivastatin, pravastatin and rosuvastatin. It is less clear whether simvastatin is transported by OATP1B1.

It seems that the inactive parent drug (simvastatin lactone) is not, but the active acid form (formed through non-enzymatic and carboxylesterase-mediated conversion within the plasma, liver and intestinal mucosa) is an OATP1B1 substrate (11, 12).

OATP1B1 is encoded by the *SLCO1B1* gene. This gene is highly expressed in the liver and may be considered a potential determinant of the inter-individual variability in statin response because it plays an important role in statin pharmacokinetics and pharmacodynamics (13). Several single nucleotide polymorphisms (SNPs) and other sequence variations have been described in *SLCO1B1* (14, 15), some of them associated with altered transport in vivo and in vitro. Among the SNPs identified the c.388A>G (Asn130Asp), c.463C>A (Pro155Thr) and c.521T>C (Val174Ala) and their derived haplotypes were the focus of several investigations of statin transport, efficacy and tolerance (12).

These SNPs have large differences in allele frequencies between different continental groups. The 521 C allele has a high frequency in Europe (14%–23%) and a lower prevalence in Africans and Asians (lower than 10%). The 388 G allele was over 60% in Sub-Saharan Africa and Asia but its frequency in Europe is lower than 50%. The 463 A allele was observed with frequencies below 10% in Sub-Saharan Africa and Asia. In Europe this allele was over 13%. Haplotypes **5* and **15* have a combined frequency of approximately 15%–20% in Europeans, 10%–15% in Asians and 2% in sub-Saharan Africans. The **1B* haplotype has a frequency of approximately 26% in Europeans, 39% in South/Central Asians, 63% in East Asians and as high as 77% in sub-Saharan Africans (8, 16). Except for small native populations from South America, no information is available about these SNPs in this continent (8).

A genome wide study from the SEARCH collaborative group showed an increase of 0.62%±1.8% reduction in LDL-C per copy of 388G allele in patients treated with 40 mg simvastatin daily (17). This study also showed that carriers of 521C allele taking 80 mg of simvastatin were found to be at a significantly increased risk of myopathy (odds ratio 4.5; 95% confidence interval 2.6–7.7). This risk further increased among homozygous CC individuals (odds ratio 16.9; 95% confidence interval 4.7–61.1). Another study expanded this association to the most common statin-induced side-effects (e.g., myalgia or muscle ache without significant creatinine kinase elevations) in a population treated with various statins (18).

In vitro studies with the 388G variant have yielded partially controversial results, some studies finding decreased activity (10, 19, 20), some finding increased activity (10, 21) and many finding no change in transport activity (10, 14, 21–26). The c.521T>C SNP was associated with markedly reduced statin transport in vitro (14, 21, 23–25), while in vivo studies showed an increased statin plasma concentration (11, 15, 27–31). The heterozygous genotype (521TC) was associated with reduced cholesterol-lowering efficacy in patients using several different statins (32), however studies with pravastatin treatment show conflicting results about this genotype influence (33, 34). A decrease of 1.28%±0.25% reduction in LDL cholesterol per copy of 521C allele in patients treated with simvastatin was observed by a genome wide study (17). In a recent study, a higher reduction in LDL-C concentrations was found in 463A homozygous individuals as compared with other genotypes at the c.463C>A site in elderly hypercholesterolemic-treated with fluvastatin (35).

In a previous study from our group (36) we reported evidences for the association of *ABCB1* gene variation and the efficacy of simvastatin treatment. Considering the multifactorial nature of statin response and the absence of information about *SLCO1B1* c.388A>G, c.463C>A and c.521T>C (Val174Ala) SNPs and their derived haplotypes in the Brazilian population, in the present study, we evaluated the distribution of *SLCO1B1* SNPS and haplotypes in this population of European ancestry and we hypothesized that genetic differences in this transporter gene could contribute to the variable response of simvastatin treatment in this population not completely explained in our previous study.

Materials and methods

Study population

Two hundred and sixteen hypercholesterolemic patients of European descent were prospectively investigated in Porto Alegre, Brazil. Part of the sample and the study design were previously described (36). For this prospective study, all patients were screened by physical examination, medical history and clinical laboratory evaluation and were invited to participate in this study. Exclusion criteria included the following: triglyceride concentration of 4.52 mmol/L or greater, unstable or uncontrolled clinically significant disease, uncontrolled hypothyroidism or diabetes and impaired hepatic or renal function. None of the patients were undergoing previous cholesterol-lowering therapy with statins or other drugs. Subjects were treated with 20 mg/day simvastatin (Zocor; Merck Sharp & Dohme, São Paulo, Brazil) for 6 months. Plasma lipid and lipoprotein levels were measured at baseline and after 2 and 6 months of treatment. The main characteristics of the sample are presented in Table 1. The subjects in the study were aged between 25 and 87 years (61.8±10.5 years) and 30.6% were males. Most patients never smoked (81.9%), some patients were ex-smokers (8.8%) and few patients were smokers (9.3%). Only 30.7% of subjects had prior cardiovascular disease, 71.8% of subjects had hypertension and 19.6% of subjects had diabetes. Patients were maintained on other medications throughout the study with no change, most used drugs were calcium channel blockers (15.3%), beta-blockers (34.7%), diuretics (40.3%) and other anti-hypertensive therapy (22.7%). The Ethics Committee of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil) approved the study protocol. All participants in the study gave written informed consent to participate in the study.

Biochemical analysis

Blood samples were collected from subjects after 12 h fasting. Total cholesterol, HDL-C, triglycerides and glucose concentrations were determined by conventional enzymatic methods. LDL-C was calculated according to Friedewald et al. (37).

Table 1 Demographic and key clinical characteristics of patients.

Characteristics	
Number	216
Age, years	61.8±10.5
Sex, % male	30.6
BMI, kg/m^2	27.8±4.6
Smoking	
Never, %	81.9
Past, %	8.8
Current, %	9.3
Prior CHD, %	30.7
Family history of CHD, %	23.0
Hypertension, %	71.8
Diabetes, %	19.6
Post-menopausal, %	56.8
Hormone therapy use, %	21.2
Glucose, mmol/L	5.5±1.38
Concomitant therapies	
ACE inhibitor, %	22.7
β-Blocker, %	34.7
Calcium channel blocker, %	15.3
Diuretics, %	40.3

Values for age, BMI and glucose concentrations are expressed as mean±SD. CHD, coronary heart disease; ACE, angiostensin-converting enzyme.

Genotype analysis

Genomic DNA was isolated from peripheral blood by standard procedures (38). SNPs c.388A>G (rs2306283), c.463C>A (rs11045819) and c.521T>C (rs4149056) at *SLCO1B1* gene were determined by allelic discrimination with Taqman 5′-nuclease assays. Genotyping for c.388A>G (ID: C_1901697_20) and c.521T>C (ID: C_30633906_10) SNPs were performed with validated TaqMan genotyping assays (Real Time PCR, Applied Biosystems, CA, USA) according to the manufacturer's recommended protocol. The c.463C>A polymorphism was genotyped with a custom genotyping assay by design (Applied Biosystems, CA, USA) according to the manufacturer's recommended protocol. The primers used for the c.463C>A assay were: forward TCAACATCGACCTTATCCACTTGTTTAATT and reverse GAAGACTTTTTACTGTCAATATTAATTCTTACCTTTTCC; the probes were VIC: CACTATCTCAGTTGATGCT and FAM: CTATCTCAGGTGATGCT. Genotyping for *ABCB1*, *CYP3A4* and *CYP3A5* polymorphisms were already reported in our previous study for 146 patients (36). The remaining 70 individuals not included in that investigation were genotyped with validated TaqMan genotyping assays (Real Time PCR, Applied Biosystems, CA, USA) according to the manufacturer's recommended protocol.

Statistical analysis

Allele frequencies were estimated by gene counting. Deviation from Hardy-Weinberg equilibrium was assessed by χ^2-tests. Haplotype frequencies and linkage disequilibrium were estimated with the Multiple Locus Haplotype Analysis program (version 3.0) (39). Continuous variables were expressed as mean with 95% confidence interval. HDL-C and triglyceride levels were log-transformed before analyses because of its skewed distribution; back transformed values are presented in results. To determine the association of the genotypes and haplotypes with response to simvastatin treatment, mean change percentage in plasma lipid levels among genotypes were compared by an analysis of covariance using a General Linear Model with type III sums of squares. This sum of squares applies to unbalanced study designs and quantifies the effect of an independent variable after adjustment for all other variables included in the model. Age, gender, smoking status, hypertension, prior cardiovascular disease, diabetes and baseline lipid concentrations, co-medication as well as *ABCB1*, *CYP3A4* and *CYP3A5* genotypes were included in each model as covariates. Statistical analysis was performed using the SPSS18.0 statistical package for Windows®. The false discovery rate procedure (FDR) was performed for multiple testing corrections using WINPEPI Software Version 11.4.

Results

Treatment with 20 mg per day of simvastatin significantly reduced the plasma concentrations of total cholesterol (–27.8%, p<0.001) and LDL-C (–38.6%, p<0.001). Triglyceride concentrations were modestly reduced (–16.6%, p<0.001), whereas the increase in HDL cholesterol did not reach statistical significance (1.08%, p=0.40).

Allele frequencies for *SLCO1B1* c.388A>G, c.463C>A and c.521T>C, *ABCB1* 1236 C>T, 2677 G>A/T, 3435 C>T, *CYP3A4* -392A>G and *CYP3A5* 6896A>G SNPs are shown in Table 2 and derived haplotypes for *SLCO1B1* and *ABCB1* in Table 3. Genotypes did not show statistically significant differences compared with those expected under Hardy-Weinberg equilibrium. Haplotypes derived from the *SLCO1B1* and *ABCB1* SNPs are shown in Table 2. Five different *SLCO1B1* haplotypes were observed: **1a*, **1b*, **5*, **14* and **15*. The 463A allele showed complete linkage disequilibrium with 388G and 521T (D′=1.000 and p<0.001). The 388G allele was in linkage disequilibrium with the 521T allele (D′=0.737, p<0.001).

The baseline, post-treatment and mean change percentage in lipid and lipoprotein concentrations after simvastatin treatment, according to *SLCO1B1* genotypes, are shown in Table 4. No associations with baseline mean plasma lipid parameters were observed. After adjustment for covariates, including *ABCB1* 1236 C>T, 2677 G>A/T, 3435 C>T, *CYP3A4* -392A>G and *CYP3A5* 6896A>G SNPs, a higher LDL cholesterol reduction after 6-month treatment was observed in patients with 388AG and 388GG genotypes when compared with 388AA homozygotes (–38.4% vs. –30.5% and –40.2% vs. –30.5%, p=0.011). Carriers of the *SLCO1B1* 388G allele had a greater reduction of total cholesterol and LDL cholesterol with simvastatin treatment, when compared with 388A homozygotes (–28.8% vs. –15.8%, p=0.005 and –39.6% vs. –30.6%, p=0.003; respectively). If the FDR for multiple comparisons is applied, these findings are no longer significant (p=0.08 for the co-dominant model and p=0.06 for the dominant model). No significant differences were observed for c.463C>A and c.521T>C SNPs on lipid and lipoproteins concentrations.

The *SLCO1B1* haplotypes showed no statistically significant differences in mean percentage reductions in lipid and lipoprotein concentrations after simvastatin treatment (Table 5). Nevertheless carriers of *SLCO1B1*1b*,

Table 2 *SLCO1B1*, *ABCB1*, *CYP3A4* and *CYP3A5* allele and genotype frequencies in the study population.

SNPs	Genotypes						Alleles	
	n	%	n	%	n	%		
SLCO1B1								
c.388A>G	AA		AG		GG		A	G
	56	25.9	111	51.4	49	22.7	0.516	0.484
c.463C>A	CC		CA		AA		C	A
	155	71.8	56	25.9	5	2.3	0.847	0.153
c.521T>C	TT		TC		CC		T	C
	152	70.4	59	27.3	5	2.3	0.84	0.16
ABCB1								
1236C>T	TT		TC		CC		T	C
	44	20.8	106	50.0	62	29.2	0.458	0.542
2677G>A/T	GG		G/NonG		NonG/NonG		G	NonG
	60	28.2	103	48.4	50	23.4	0.523	0.477
3435C>T	TT		TC		CC		T	C
	62	29.0	101	47.2	51	23.8	0.526	0.474
CYP3A4								
-392A>G	*1B/*1B		*1B/*1A		*1A/*1A		*1B	*1A
	201	96.2	8	3.8	0	0	0.981	0.019
CYP3A5								
6896A>G	*1/*1		*1/*3		*3/*3		*1	*3
	1	0.5	38	18.2	170	81.3	0.096	0.904

212 individuals were genotyped for *ABCB1* 1236 C>T, 213 for *ABCB1* 2677 G>A/T, 214 for *ABCB1* 3435 C>T and 209 for CYP3A4/3A5 genes. From those, 146 were previously described [36].

*SLCO1B1*14* and *SLCO1B1*15* haplotypes containing the 388G allele showed a greater reduction in total cholesterol and LDL cholesterol, respectively, when compared with *SLCO1B1*1a* haplotype. These results suggest that the difference lipid levels reduction is due to the 388G allele effect, it did not reach threshold for statistical significance in haplotype analyses because the 388G carriers were divided into three different haplotypes.

Discussion

In this study, we examined the possible influence of common polymorphisms in *SLCO1B1* gene and the efficacy of simvastatin 20 mg/day treatment. Our major finding was the 388G allele association with simvastatin response. Indeed, in the investigated sample, this genetically determined response confers a gain of 9% in LDL-C reduction in homozygous 388GG

Table 3 Haplotypes frequencies of *SLCO1B1* and *ABCB1* genes in the study population.

Haplotypes				N chromosomes	Frequency[a]
SLCO1B1	c.388A>G	c.463C>A	c.521T>C		
SLCO1B1*1a	A	C	T	216	0.50
SLCO1B1*1b	G	C	T	81	0.19
SLCO1B1*5	A	C	C	7	0.02
SLCO1B1*14	G	A	T	66	0.15
SLCO1B1*15	G	C	C	62	0.14
ABCB1	1236C>T	2677G>A/T	3435C>T		
	C	G	C	181	0.43
	T	NonG	T	176	0.41
	C	NonG	T	12	0.03
	T	G	T	5	0.01
	C	G	T	30	0.07
	C	NonG	C	7	0.02
	T	G	C	7	0.02
	T	NonG	C	6	0.01

[a]Frequencies were estimated with 216 individuals for *SLCO1B1* haplotypes and 212 individuals for *ABCB1* haplotypes. *ABCB1* haplotypes from 146 patients were previously described [36].

Table 4 Baseline lipid and lipoprotein levels and mean change percentage after simvastatin treatment according to *SLCO1B1* polymorphisms.

SLCO1B1	n	Baseline, mmol/L				Post-treatment, mmol/L				Mean change, %[a]			
SNPs		TC	LDL-C	HDL-C	TG	TC	LDL-C	HDL-C	TG	TC	LDL-C	HDL-C	TG
c.388A>G													
AA	56	6.45	4.35	1.28	1.58	4.83	2.87	1.31	1.30	–15.3	–30.5	0.13	–23.3
		6.1:6.7	4.0:4.6	1.2:1.3	1.4:1.7	4.5:5.0	2.6:3.1	1.2:1.3	1.1:1.4	–26.0:–4.5	–39.7:–21.3	–10.6:10.9	–51.0:4.3
AG	111	6.66	4.56	1.24	1.69	4.76	2.74	1.26	1.42	–20.7	–38.4	–3.3	–23.0
		6.4:6.8	4.3:4.7	1.1:1.3	1.5:1.8	4.6:4.9	2.6:2.8	1.2:1.3	1.3:1.5	–30.8:–10.7	–46.0:–30.8	–12.4:5.6	–48.9:2.8
GG	49	6.74	4.67	1.28	1.59	4.75	2.75	1.26	1.39	–22.4	–40.2	–2.3	–23.0
		6.4:7.0	4.4:4.6	1.2:1.3	1.3:1.8	4.4:5.0	2.5:2.9	1.1:1.3	1.2:1.5	–32.2:–12.5	–48.4:–31.9	–12.1:7.4	–48.6:2.6
ANCOVA										**0.014**	**0.011**	0.56	0.92
AA	56	6.45	4.35	1.28	1.58	4.83	2.87	1.31	1.30	–15.8	–30.6	0.18	–23.3
		6.1:6.7	4.0:4.6	1.2:1.3	1.4:1.7	4.5:5.0	2.6:3.1	1.2:1.3	1.1:1.4	–26.4:–5.1	–39.7:–21.4	–10.5:10.9	–50.8:4.1
AG+GG	160	6.68	4.59	1.25	1.66	4.75	2.75	1.26	1.41	–28.8	–39.0	–3.0	–23.0
		6.5:6.8	4.4:4.7	1.2:1.3	1.5:1.7	4.6:4.8	2.6:2.8	1.2:1.3	1.3:1.5	–31.4:–12.0	–46.3:–31.7	–11.6:5.6	–48.1:2.0
ANCOVA										**0.005**	**0.003**	0.30	0.95
c.463C>A													
CC	155	6.59	4.51	1.25	1.63	4.78	2.81	1.26	1.36	–21.8	–37.3	–3.9	–25.5
		6.4:6.7	4.3:4.6	1.2:1.2	1.5:1.7	4.6:4.9	2.6:2.9	1.2:1.3	1.2:1.4	–31.8:–11.8	–45.1:–29.4	–13.0:5.1	–50.7:–0.3
CA	56	6.69	4.57	1.26	1.74	4.74	2.69	1.30	1.47	–23.0	–41.3	–1.6	–17.4
		6.3:7.0	4.2:4.8	1.1:1.3	1.5:1.9	4.4:4.9	2.4:2.9	1.2:1.3	1.3:1.6	–33.0:–12.8	–49.4:–33.2	–11.0:7.8	–43.3:8.4
AA	5	6.75	4.77	1.40	1.09	4.79	2.87	1.35	1.12	–24.1	–40.0	–4.7	–18.7
		5.2:8.2	3.7:5.7	0.9:2.0	0.6:1.9	3.7:5.8	2.1:3.5	0.9:1.8	0.8:1.5	–38.8:–9.4	–56.7:–23.4	–24.4:14.9	–56.2:18.7
ANCOVA										0.79	0.33	0.76	0.30
CC	155	6.59	4.51	1.25	1.63	4.78	2.81	1.26	1.36	–21.8	–37.3	–3.7	–25.5
		6.4:6.7	4.3:4.6	1.2:1.2	1.5:1.7	4.6:4.9	2.6:2.9	1.2:1.3	1.2:1.4	–31.8:–11.8	–45.1:–29.6	–12.8:5.2	–50.5:–0.4
CA+AA	61	6.70	4.58	1.27	1.67	4.75	2.71	1.30	1.44	–23.1	–41.3	–1.7	–17.5
		6.4:7.0	4.3:4.8	1.1:1.3	1.4:1.9	4.5:4.9	2.5:2.9	1.2:1.3	1.2:1.6	–33.3:–12.9	–49.3:–33.2	–11.1:7.6	–43.2:8.2
ANCOVA										0.51	0.13	0.50	0.12
c.521T>C													
TT	152	6.61	4.51	1.26	1.64	4.78	2.77	1.29	1.41	–21.0	–38.6	–1.5	–16.3
		6.4:67	4.3:4.6	1.2:1.3	1.5:1.7	4.6:4.9	2.6:2.8	1.2:1.3	1.3:1.5	–31.1:–10.9	–46.0:–30.6	–11.5:6.3	–41.8:–9.1
TC	59	6.63	4.55	1.25	1.65	4.79	2.80	1.25	1.33	–22.7	–39.9	–1.9	–26.7
		6.3:6.9	4.3:4.8	1.1:1.3	1.4:1.8	4.5:4.9	2.6:2.9	1.1:1.3	1.1:1.5	–32.8:–12.6	–48.5:–31.3	–11.9:7.9	–52.0:–1.4
CC	5	6.80	4.83	1.17	1.65	4.54	2.80	1.13	1.26	–27.4	–42.1	–10.9	–34.0
		6.1:7.4	4.2:5.4	1.0:1.3	1.0:2.5	3.2:5.8	1.8:3.7	0.8:1.4	0.9:1.6	–41.6:–13.7	–57.9:–26.3	–29.5:7.5	–69.8:1.7
ANCOVA										0.40	0.77	0.67	0.07
TT	152	6.61	4.51	1.26	1.64	4.78	2.77	1.29	1.41	–28.1	–38.4	–2.8	–16.4
		6.4:6.7	4.3:4.6	1.2:1.3	1.5:1.7	4.6:4.9	2.6:2.8	1.2:1.3	1.3:1.5	–31.1:–11.0	–46.0:–30.7	–11.7:6.0	–41.8:9.0
TC+CC	64	6.65	4.57	1.24	1.65	4.77	2.80	1.24	1.33	–23.2	–40.2	–3.1	–27.4
		6.4:6.9	4.3:4.8	1.1:1.3	1.4:1.8	4.5:4.9	2.6:2.9	1.1:1.3	1.1:1.4	–33.2:–13.2	–48.6:–31.8	–12.8:6.6	–52.5:–2.4
ANCOVA										0.28	0.44	0.89	0.02

[a]Mean change percentage adjusted for all covariates in the model: age, gender, smoking status, hypertension, prior cardiovascular disease, diabetes, baseline lipid concentrations, *CYP3A4* -392A>G genotypes, *CYP3A5* 6986A>G genotypes, *ABCB1* genotypes (11236C>T, 2677G>A/T and 3435C>T) and drugs administered in concomitant therapies. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides. Values are expressed as mean and 95% confidence interval. Bold values are significant p-values.

as compared to homozygous wild-type 388AA individuals. The heterozygous individuals presented intermediate values (7%).

The global analyses reported by Pasanen et al. (16) showed that the c.521T>C SNP varied markedly between populations. The lowest c.521C frequencies were observed in Sub-Saharan Africans and the highest in Native American populations. In contrast, the c.388G allele at the c.388A>G SNP had a higher prevalence in Africa. Pasanen et al. (16) have also shown that the *SLCO1B1*1b* haplotype was the most common haplotype in African and Native American populations, whereas haplotype **5* was only seen in Europe and the Middle East. The observed allele and haplotype frequencies of the polymorphisms tested in this Southern Brazilian population were in the same range as those reported in other European or European-derived populations (8, 13).

The wide inter-individual response to statins is well established. It has been suggested that genetic differences in hepatic transporters may alter the exposure of statins to their site of action, altering their cholesterol-lowering ability. SLCO1B1 mediates the transport of statins into hepatocytes. Such uptake determines both intra-hepatocyte and residual circulating statin concentrations and potentially constitutes one of the rate-limiting steps in the action of this class of drug (12). In vitro pharmacokinetic studies suggest that, despite

Table 5 Baseline lipid and lipoprotein levels and mean change percentage after simvastatin treatment according to *SLCO1B1* haplotype.

Haplotypes	n	Baseline, mmol/L				Post-treatment, mmol/L				Mean change, %[a]			
		TC	LDL-C	HDL-C	TG	TC	LDL-C	HDL-C	TG	TC	LDL-C	HDL-C	TG
*1a/*1a	62	6.55	4.35	1.30	1.70	4.83	2.86	1.30	1.32	–20.6	–27.7	–3.3	–11.3
		6.2:6.9	4.1:4.6	1.2:1.4	1.5:1.9	4.5:5.1	2.6:3.1	1.2:1.4	1.2:1.5	–28.7:–12.4	–38.8:–16.6	–16.2:9.4	–31.9:9.2
*1a/*1b	44	6.60	4.53	1.28	1.56	4.78	2.74	1.29	1.45	–25.1	–34.2	–9.1	–2.9
		6.4:6.8	4.3:4.7	1.2:1.4	1.4:1.7	4.6:5.0	2.5:3.0	1.2:1.4	1.3:17	–32.9:–17.3	–44.8:–23.6	–21.6:3.3	–23.0:17.1
*1b/*1b	14	6.99	4.78	1.27	1.87	4.82	2.87	1.22	1.46	–28.6	–36.0	–9.6	–15.2
		6.4:7.6	4.3:5.3	1.1:1.4	1.5:2.4	4.3:5.4	2.4:3.4	1.1:1.3	1.1:1.9	–37.8:–19.4	–48.5:–23.5	–24.2:4.9	–38.5:8.0
*1a/*5	5	6.06	4.13	1.27	1.22	4.54	2.82	1.21	1.03	–14.5	–18.2	–3.2	–4.9
		4.5:7.6	2.9:5.4	1.0:1.6	0.6:2.6	3.7:5.4	2.0:3.6	0.9:1.7	0.6:1.8	–31.1:1.9	–40.4:–3.9	–29.0:22.5	–37.3:47.1
*1a/*14	42	6.61	4.46	1.23	1.77	4.70	2.63	1.25	1.51	–25.7	–36.7	–6.1	–1.9
		6.2:7.0	4.1:4.8	1.1:1.4	1.5:2.1	4.4:5.0	2.4:2.9	1.1:1.4	1.3:1.8	–33.5:–17.9	–47.3:–26.0	–18.5:6.2	–21.7:17.8
*14/*14	6	6.76	4.77	1.40	1.09	4.70	2.87	1.35	1.12	–26.5	–35.1	–7.5	–3.3
		5.2:8.3	3.8:5.7	1.0:2.0	0.6:1.9	3.8:5.8	2.2:3.6	1.0:1.9	0.8:1.6	–39.8:–13.3	–53.1:–17.1	–28.7:13.6	–37.3:30.7
*1a/*15	39	6.76	4.63	1.24	1.84	4.71	2.76	1.24	1.34	–27.5	–35.9	–5.1	–24.6
		6.4:7.1	4.3:4.9	1.2:1.3	1.6:2.2	4.5:5.0	2.6:3.0	1.2:1.3	1.1:1.6	–35.5:–19.5	–47.1:–24.8	–17.8:7.6	–45.2:–4.1
*15/*15	4	6.63	4.79	1.09	1.54	4.51	2.67	1.14	1.45	–29.3	–38.2	–7.7	–14.5
		5.2:8.1	3.7:5.9	0.8:1.5	1.0:2.5	0.8:8.2	0.0:5.4	0.5:2.4	0.9:2.2	–44.7:–13.8	–59.2:–17.1	–32.5:16.9	–53.9:24.7
ANCOVA										0.23	0.26	0.94	0.11

[a]Mean change percentage adjusted for all covariates in the model: age, gender, smoking status, hypertension, prior cardiovascular disease, diabetes, baseline lipid concentrations, *CYP3A4* -392A>G genotypes, *CYP3A5* 6986A>G genotypes, *ABCB1* genotypes (11236C>T, 2677G>A/T and 3435C>T) and drugs administered in concomitant therapies. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides. Values are expressed as mean and 95% confidence interval.

a large number of detected polymorphisms at the *SLCO1B1* gene, only two relatively common, non-synonymous SNPs (c.388A>G and c.521T>C) showed altered transport function. In vivo pharmacokinetic studies support these findings and suggest that a single copy of either variant is sufficient to increase statin plasma concentration (40, 41). Several studies reported that the c.521T>C polymorphism affects the uptake of statins (15, 27, 28, 30). In one such work it has been shown that the carriers of the 521C allele have a reduction in simvastatin acid form uptake (11). Japanese carriers of this allele have a reduced efficacy to treatment with statins, including simvastatin (33). The SEARCH collaborative group showed attenuated response in LDL cholesterol reduction per copy of 521C allele in patients treated with simvastatin (17). Despite such evidences, in our study, carriers of the 521C allele did not have an influence on response to treatment with simvastatin. The c.463C>A SNP, which recently was associated with response to fluvastatin treatment in elderly hypercholesterolemia (35), did not show differences between genotypes of patients treated with simvastatin in the present study.

So far, the polymorphisms of OATP1B1 appear to influence statin response in different ways. The c.463C>A SNP and *SLCO1B1*14* haplotype are associated with enhanced efficacy to fluvastatin treatment (35), c.521T>C seems to be associated with an attenuated lipid-lowering efficacy of pravastatin (32, 33) and simvastatin (17) in some investigations, but not all (34), and this study suggests an influence of the 388G allele in simvastatin treatment response in agreement with the SEARCH collaborative group (17). Although the data presented here and the magnitude of the effect in the present study appears to be inconsistent with previous investigations, the overall direction did not differ and some variables may explain these apparent discrepancies. First, despite a similar mechanism of action for all statins, metabolism and drug interactions could vary considerably among them. Differences in genetic variations in drug metabolizing enzymes and other transporters and even in other genes associated with lipid metabolism may be important to explain these differences. Takane et al. (42) pinpointed the importance of determining the time point when on-treatment cholesterol data were obtained in these pharmacodynamics studies. They reported a significantly attenuated LDL-C lowering in carriers of haplotype* 15 after 8 weeks of treatment. However, this association was lost when the analysis was repeated after 1 year. They suggested therefore that *SLCO1B1* haplotypes may be predictive of a slower rather than a prolonged attenuated response to statin therapy. These results taken together suggest that OATP1B1 polymorphisms investigations developed with a specific statin may not be extrapolated to all kinds of statins. Only further studies of OATP1B1 variants with each statin may help elucidate the role of these uptake carriers in HMG-CoA reductase inhibitors efficacy.

An important limitation of the present study was the moderate sample size. The possibility of type II error could not be excluded because the power to detect this association was only about 60%. We performed the False Discovery Rate procedure for multiple testing corrections. However, when the probability of the type I error (false positive) decreases, the probability of the type II error (false negative) increases. Since adjustment for multiple testing is not consensual (43), we show both p-values (corrected and not corrected). Since there is nominal significance for association and the overall direction is the same as reported in the literature, the data presented herein strengthen that the c.388A>G variant is associated with

LDL-C lowering. Although it is not possible to exclude that the effects we observed were due to lack of adherence to treatment, there is no reason to expect a preferential compliance to simvastatin according to the presence of *SCLO1B1* genotypes. The discrepancies among investigations might be the result of type I and type II errors, differences among study designs, phenotypic differences between responders and non-responders, number of individuals investigated and/or other distinct methodological approaches. Apart from heterogeneity in the methodology among studies, the inconsistent findings may also be related to the complex nature of the phenotype of statin response. It might also have been exposed to factors, such as population stratification, but previous investigations have shown an absence of genetic sub-structure in this southern Brazilian population (44). Despite these limitations, the major strength of this investigation is its prospective character and all patients entering the study protocol were on the same dose (20 mg/day) of the same statin (simvastatin).

In conclusion, the present study suggests that the *SLCO1B1* c.388A>G gene polymorphism could play a role in the inter-individual variation of clinical response to simvastatin in Brazilians. These results add to those that suggest that the effects of *SLCO1B1* variants may be substrate-specific. However, additional studies and independent replication in different cohorts are essential before extension of data for clinical practice will be possible.

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Impact of population diversity on the prediction of 7-SNP NAT2 phenotypes using the tagSNP rs1495741 or paired SNPs

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A novel *NAT2* tagSNP (rs1495741) and a 2-SNP genotype (rs1041983 and rs1801280) have been recently shown to accurately predict the NAT2 acetylator phenotypes in populations of exclusive or predominant European/White ancestry. We confirmed the accuracy of the tagSNP approach in White Brazilians, but not in Brown or Black Brazilians, sub-Saharan Mozambicans, and Guarani Amerindians. The combined rs1041983 and rs1801280 genotypes provided considerably better prediction of the NAT2 phenotype in Guarani, but no consistent improvement in Brown or Black Brazilians and Mozambicans. Best predictions of the NAT2 phenotype in Mozambicans using *NAT2* SNP pairs were obtained with rs1801280 and rs1799930, but the accuracy of the estimates remained inadequate for clinical use or for investigations in this sub-Saharan group or in Brazilians with considerable African ancestry. In conclusion, the rs1495741 tagSNP cannot be applied to predict the NAT2 acetylation phenotype in Guarani and African-derived populations, whereas 2-SNP genotypes may accurately predict NAT2 phenotypes in Guarani, but not in Africans. *Pharmacogenetics and Genomics* 00:000–000

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Introduction

The *NAT2* gene, which encodes the enzyme arylamine *N*-acetyltransferase-2 (NAT2), exhibits single nucleotide polymorphisms (SNPs) in human populations that modify the metabolism of prescribed medicines and other xenobiotics, including carcinogens. Genotyping of seven of these SNPs, namely rs1801279 (191G > A), rs1041983 (282C > T), rs1801280 (341T > C), rs1799929 (481C > T), rs1799930 (5980G > A), rs1208 (803G > A), and rs1799931 (857G > A), is recommended for inferring the NAT2 acetylation phenotype in various populations. Two recent articles in *Pharmacogenetics and Genomics* assessed the accuracy of a novel *NAT2* tagSNP, namely rs1495741, to predict the 7-SNP-inferred phenotypes in populations of exclusive [1] or predominant [2] European ancestry. In both studies, the tagSNP and the 7-SNP genotype showed a high degree of correlation, but Selinski *et al.* [2] observed that the 7-SNP genotype outperformed the tagSNP with respect to the specificity of NAT2 phenotype inference. These authors further reported that a 2-SNP genotype (rs1041983 and rs1801280) previously identified by Cascorbi *et al.* [3] predicted NAT2 phenotypes with similar specificity and sensitivity as the conventional 7-SNP genotype. In the present communication, we examined the accuracy of the tagSNP and of distinct 2-SNP genotypes to predict the 7-SNP-inferred NAT2 phenotype in populations that are frequently under-represented in pharmacogenomic databases, namely Amerindians (Native Americans) and sub-Saharan Africans. In addition, we present data for an admixed Brazilian cohort, with African, European, and Amerindian ancestral roots.

Materials and methods

Study population

Amerindians were represented by 88 healthy, adult Guarani (Ñandeva and Kaiowá subgroups) who were contacted in indigenous reservation areas of Amanbaí, Limão Verde, and Porto Lindo, state of Mato Grosso do Sul, in Brazil's center–west region, in the context of a study of population genetics of Amerindian groups, approved by the Brazilian National Ethics Committee [4]. Non-Amerindian, healthy, adult Brazilians, self-identified as White ($n = 136$), Brown ('pardo', in Portuguese, $n = 107$), and Black ($n = 118$), according to the Brazilian Census Classification (*www.ibge.gov.br*), were recruited in the cities of Rio de Janeiro and Porto Alegre, in Brazil's south-east and south regions, respectively. The study protocol was approved by the Ethics Committees of the Instituto Nacional de Câncer, Rio de Janeiro. The sub-Saharan cohort included 103 adult Mozambicans (36 healthy individuals and 67 cardiovascular

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patients under warfarin therapy), recruited in Maputo, as part of a study approved by the Ethics Committee of the Universidade Eduardo Mondlane, Maputo, Mozambique.

Genotyping and statistics

Allele discrimination at eight polymorphic *NAT2* loci (rs1801279, rs1041983, rs1801280, rs1799929, rs1799930, rs1208, rs1799931, and the tagSNP, rs1495741) was performed on a Fast 7500 Real-Time System (Applied Biosystems, Foster City, California, USA) using validated Taqman assays. Allele and genotype frequencies at each locus were derived by gene counting. Deviations from Hardy–Weinberg equilibrium were assessed using the goodness-of-fit χ^2-test. Calculation and visualization of pair-wise linkage disequilibrium (LD) between the SNPs were carried out using the software Haploview version 4.1 [5]. 7-SNP (rs1041983, rs1801280, rs1799929, rs1799930, rs1208, and rs1799931) *NAT2* haplotypes and acetylation phenotypes were inferred as described in *http://www.louisville.edu/medschool/pharmacology/NAT.html*. Individuals were classified as rapid, intermediate, and slow acetylators if they possessed zero, one, or two slow acetylation *NAT2* alleles, namely *NAT2*5*, *NAT2*6*, *NAT2*7*, and *NAT2*14*. Inference of the NAT2 acetylation phenotype from 2-SNP genotypes was based on the sum of variant alleles at each pair of *NAT2* polymorphic sites examined. Individuals with zero, one, or at least two reduced activity alleles were classified as rapid, intermediate, and slow acetylators, respectively [2]. Contingency tables were used to visualize the agreement between the inferred 7-SNP phenotypes and either the tagSNP (rs1495741) or the 2-SNP genotypes.

Results

Genotype distribution and minor allele frequency for the eight SNPs genotyped in the five population groups are shown in Supplemental Table 1, *http://links.lww.com/FPC/A392*. The only significant deviation from Hardy–Weinberg equilibrium ($P = 0.028$) was in relation to rs1041983 in White Brazilians. Supplemental Table 2, *http://links.lww.com/FPC/A393* presents the r^2 values for pair-wise LD between the genotyped SNPs in the study groups. The seven SNPs and the tagSNP are located within the same block but the degree of LD differs among populations (Supplemental Figs 1–5, *http://links.lww.com/FPC/A391*), with the highest LD in White Brazilians and the lowest in Mozambicans and Black Brazilians. The 7-SNP-inferred acetylator phenotypes are displayed in Supplemental Table 3, *http://links.lww.com/FPC/A394*.

The accuracy of the tagSNP rs1495741 genotype to predict the 7-SNP-inferred phenotype varied considerably among the study groups, decreasing stepwise from White to Brown and then to Black Brazilians and Guarani, and showing the poorest performance in Mozambicans. This trend is evident in the percent of correctly predicted phenotypes and in the sensitivity of the tagSNP AA genotype as a predictor of the 7-SNP-inferred slow phenotype (Table 1). In addition, the specificity of the tagSNP genotypes to predict the NAT2 acetylator phenotype had the lowest value in Mozambicans (0.85). Misclassification for the extreme categories (i.e. tagSNP AA genotype vs. 7-SNP rapid acetylators or tagSNP GG genotype vs. 7-SNP slow acetylators) was not observed in White or Black Brazilians and occurred rarely (< 3%) in the other groups. Misclassification between the tagSNP AA genotype and the 7-SNP intermediate acetylators did not occur in White Brazilians or Guarani, but was observed in 2–7.8% of individuals in the other three groups. Other forms of misclassification were considerably more common, ranging in frequency from 6.7% in White Brazilians to 19.2% in Guarani (Table 1).

Prompted by the study of Selinski *et al.* [2], we next examined the accuracy of the 2-SNP genotype on the basis of 282C > T and 341T > C, to predict the 7-SNP-inferred NAT2 phenotypes (Table 2). The 2-SNP genotype outperformed the tagSNP in White Brazilian and especially in Guarani, in whom the 2-SNPs correctly predicted 97.7% of the 7-SNP haplotypes, with a sensitivity of 0.96 and a specificity of 1.0. The corresponding values for the tagSNP in Guarani were 79.5%, 0.744, and 1.0. In Brown and Black Brazilians, the 2-SNP genotype predicted the 7-SNP acetylator phenotype with greater sensitivity, but lower specificity than the tagSNP genotype. The percent of correctly predicted phenotypes was higher with the 2-SNP genotype in Black, but not in Brown Brazilians. In Mozambicans, the 2-SNP genotype performed poorly, with only 63.1% of correctly predicted 7-SNP NAT2 phenotypes.

The poor performance of both the tagSNP and the combined 282C > T and 341T > C genotypes in Mozambicans led us to examine all other 2-SNP genotypes as predictors of the 7-SNP NAT2 phenotypes in this cohort (Supplemental Table 4, *http://links.lww.com/FPC/A395* and Supplemental Table 5, *http://links.lww.com/FPC/A396*). Best results (84.3% correct predictions, specificity 1.0, sensitivity 0.810) were observed with the 341T > C and 590G > A SNPs. We extended the assessment of the accuracy of this genotype pair as a predictor of the 7-SNP phenotype to the other study cohorts, and observed a specificity of 1.0 in all groups, but considerable variations in the sensitivity and percent of correctly predicted phenotypes (Supplemental Table 5, *http://links.lww.com/FPC/A396*). The best performance was observed in White Brazilians, followed by Brown Brazilians, Black Brazilians, and Mozambicans; in Guarani, the 341T > C and 590G > A genotypes performed poorly, with 62.5% of correctly predicted phenotypes and a sensitivity of 0.489, reflecting the inaccuracy in identifying the 7-SNP slow acetylator phenotype.

Discussion

Human genetic diversity is evident in the frequency distribution and LD of pharmacogenetic polymorphisms

Table 1 Agreement between the NAT2 phenotype inferred from seven SNPs or with tagSNP rs1495741, in five distinct population groups

		tagSNP genotype						Misclassification (%)		
Groups (*n*)	7-SNP-inferred phenotype	GG	AG	AA	GG + AG	Sensitivity Specificity	Agreement (%)	Extreme categories	AA vs. intermediate	Other
White Brazilian (133)	Rapid	3	3	0	3	0.975 0.942	93.2	0	2.3	4.5
	Intermediate	1	42	3	43					
	Slow	0	2	79	2					
	Rapid + intermediate	4	45	3	–					
Brown Brazilian (107)	Rapid	6	1	0	7	0.881 0.913	87.6	1.0	3.8	7.6
	Intermediate	1	34	4	35					
	Slow	1	6	52	7					
	Rapid + intermediate	7	35	4	–					
Black Brazilian (99)	Rapid	6	3	0	9	0.761 0.962	80.8	0	2.0	17.2
	Intermediate	3	39	2	42					
	Slow	0	11	35	11					
	Rapid + intermediate	9	42	2	–					
Mozambicans (102)	Rapid	9	2	1	11	0.786 0.850	74.5	2.9	7.8	14.7
	Intermediate	6	34	8	40					
	Slow	2	7	33	9					
	Rapid + intermediate	15	36	9						
Guarani (77)	Rapid	3	0	0	3	0.744 1.000	80.5	1.3	0	18.2
	Intermediate	5	30	0	35					
	Slow	1	9	29	10					
	Rapid + intermediate	8	30	0	–					

Agreement indicates that individuals with tagSNP genotypes GG, AG, or AA have rapid, intermediate, or slow 7-SNP-inferred phenotypes, respectively.
Sensitivity is defined as the probability of being rs1495741 AA for individuals with the NAT2 slow inferred phenotype.
Specificity is defined as the probability of being rs1495741 = GG or AG for individuals with the NAT2 rapid or intermediate phenotypes.

Table 2 Agreement between the NAT2 phenotype inferred from seven SNPs or with 282C>T and 341T>C, in five population groups

		2-SNP genotype						Misclassification (%)		
Groups (*n*)	7-SNP-inferred phenotype	0	1	≥ 2	0 or 1	Sensitivity Specificity	Agreement (%)	Extreme categories	2-SNP>2 genotype vs. intermediate	Other
White Brazilian (136)	Rapid	7	0	0	7	1.000 0.981	99.3	0	0.7	0
	Intermediate	0	46	1	46					
	Slow	0	0	82	0					
	Rapid + intermediate	7	46	1	–					
Brown Brazilian (107)	Rapid	5	1	1	6	0.949 0.771	86.0	0.9	9.4	3.7
	Intermediate	0	31	10	31					
	Slow	0	3	56	3					
	Rapid + intermediate	5	32	11	–					
Black Brazilian (118)	Rapid	7	2	0	9	0.964 0.839	87.3	0.8	8.5	3.4
	Intermediate	1	42	10	43					
	Slow	1	1	54	2					
	Rapid + intermediate	8	44	10	–					
Mozambicans (103)	Rapid	3	8	2	11	0.857 0.689	63.1	1.9	16.5	18.5
	Intermediate	5	26	17	31					
	Slow	0	6	36	6					
	Rapid + intermediate	8	34	19	–					
Guarani (88)	Rapid	4	0	0	4	0.956 1.000	97.7	0	0	2.3
	Intermediate	0	39	0	39					
	Slow	0	2	43	2					
	Rapid + intermediate	4	39	0	–					

Agreement indicates that individuals with 2-SNP genotypes 0, 1, or at least 2 have rapid, intermediate, or slow 7-SNP-inferred phenotypes, respectively.
Sensitivity is defined as the probability of having the 2-SNP genotype at least 2 for individuals with the NAT2 slow inferred phenotype.
Specificity is defined as the probability of having the 2-SNP genotypes 0 or 1 for individuals with the NAT2 rapid or intermediate phenotypes.

of clinical relevance. As a corollary, to impact positively on global health, pharmacogenetics must adopt an inclusive population approach. The present study explores this notion with respect to the recent controversy on the accuracy of the tagSNP rs1495741 to predict the NAT2 acetylator phenotype in exclusively [1] or predominantly European cohorts [2]. For this purpose, we enrolled an Amerindian group living in Brazil (Guarani), a sub-Saharan population (Mozambican), and non-Amerindian Brazilians self-identified as White, Brown, or Black. The latter three groups differ substantially in the average proportions of African and European ancestry, despite an extensive overlap of the individual proportions of these biogeographical ancestries within each 'color' category [6,7]. The observed frequency of the *NAT2* alleles genotyped in our study and of their genotypes and inferred phenotypes are consistent with previous results in non-Amerindian Brazilians [8] and sub-Saharan African populations [9,10]. However, the frequency of the *NAT2* polymorphisms in Guarani differed from other Amerindian populations, especially in relation to the 341C slow allele, which was far more common in Guarani (0.511) than in Emberá (0.104) and Ngawbé (0.021) from Panamá [11] and other groups [12]. As a consequence, the frequency of the NAT2 slow acetylator phenotype was considerably higher in the Guarani (45%) than in combined Emberá/Ngawbé (15%, Arias *et al.* [11]) or the overall Amerindian cohort studied by Fuselli *et al.* [12]. Therefore, the results of the present study may not be applicable to other extant Native American populations.

Our results for White Brazilians support the notion that the tagSNP rs1495741 predicts with high accuracy the 7-SNP-inferred haplotype in populations of predominant White/European background [1]. Of note, we observed no misclassifications for the extreme phenotypes in White Brazilians, in agreement with data for Spaniards [1]. However, the predictive performance of the tagSNP declined stepwise from White, to Brown, and to Black Brazilians, and reached its lowest level in Mozambicans. These data are consistent with the progressive increase in the average proportion of African ancestry (and reduction in European ancestry) from White to Brown and to Black Brazilians [6,7] and, furthermore, suggest that rs1495741 does not predict accurately the NAT2 acetylation phenotype in populations of African descent. This conclusion may be extended to Guarani, in view of the relatively poor predicting performance of the rs1495741 AA genotype in this Amerindian group.

The observation that the (282C > T, 341T > C) 2-SNP genotype outperformed the tagSNP as a predictor of the NAT2 acetylation phenotype in Europeans, Venezuelan, and Pakistani cohorts [2] was verified in White Brazilians, and extended to the Guarani. The effect was more remarkable in the latter because of the relatively poor performance of the tagSNP in this Amerindian group. By contrast, the paired 282C > T and 341T > C genotypes performed poorly in Mozambicans, and did not show a consistent improvement over the tapSNP in Brown and Black Brazilians. Collectively, these data suggest to us that the combined 282C > T and 341T > C genotypes cannot replace the standard 7-SNP genotype for inference of the NAT2 acetylator phenotype in populations of African descent. Among all other pairs of SNPs genotyped in the present study, 341T > C and 590G > A provided the highest percent of correctly predicted phenotypes (84.3%) in Mozambicans, with a specificity of 1.0 and a sensitivity of 0.810. This is a considerably better performance, relative to either the tagSNP or the paired 282C > T and 341T > C SNPs, but still insufficient for replacing the standard 7-SNP-inferred NAT2 phenotype for clinical or investigational purposes in Mozambicans and possibly other sub-Saharan populations. The 341T > C and 590G > A SNP pair has been previously shown to predict the NAT2 phenotype with high specificity in Spaniards [13], and this was confirmed in both non-Amerindian Brazilians and the Guarani. However, the percent of correctly predicted phenotypes in White and Black Brazilians, and especially in Guarani using the 341T > C and 590G > A SNP pair was lower than with 282C > T and 341T > C. The relative rarity (1.7%) of the 590A allele in Guarani may account for the poor performance of the 341T > C and 590G > A SNP pair in this group.

In conclusion, we have extended the investigation of the tagSNP rs149574 as a predictor of the NAT2-inferred phenotype to Amerindian, sub-Saharan African, and admixed Brazilian cohorts. The results confirmed the accuracy of this tagSNP approach in White Brazilians, but not in Brown or Black Brazilians, Mozambicans, and Guarani. The 282C > T and 341T > C genotype pair provided considerably better prediction of the NAT2 phenotype in Guarani, but no consistent improvement in Brown or Black Brazilians and Mozambicans. Best predictions of the NAT2 phenotype in Mozambicans using *NAT2* SNP pairs were obtained with 341C > T and 590G > A, but the accuracy of the estimates appears to be inadequate for clinical use or for investigations in Mozambicans or in Brazilians with distinct African ancestry. Collectively, the data reported here emphasize the impact of population diversity on the distribution and LD of pharmacogenetic polymorphisms of clinical and toxicological relevance.

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Conflicts of interest

There are no conflicts of interest.

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Accuracy of *NAT2* SNP genotyping panels to infer acetylator phenotypes in African, Asian, Amerindian and admixed populations

A letter in response to: Hein DW, Doll MA. Accuracy of various human *NAT2* SNP genotyping panels to infer rapid, intermediate and slow acetylator phenotypes. *Pharmacogenomics* 13(1), 31–41 (2012).

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In a recent article in *Pharmacogenomics*, Hein and Doll assessed the accuracy of different genotyping panels to infer the NAT2 acetylator phenotype in cryopreserved human hepatocytes [1]. Comparison of panels based on a single tagSNP (rs1495741) or combinations of two to seven SNPs led the authors to recommend a 4-SNP genotype panel, including rs1801279 (191G>A), rs1801280 (341T>C), rs1799930 (590G>A) and rs1799931 (857G>A) for accurate inference of the NAT2 acetylator phenotypes, 'particularly in populations of non-European ancestry'. These populations, however, were scarcely represented in the Hein and Doll study, which comprised predominantly (80%) samples from Caucasian Americans [1]. Having recently shown that the accuracy of NAT2 acetylator phenotype inference based on either rs1495741 or paired *NAT2* SNPs varies markedly across continental populations [2], we decided to examine whether the conclusions proposed by Hein and Doll apply to distinct non-European cohorts, including Mozambicans from southwest Africa (n = 102), Japanese (n = 87), Guarani–Amerindians living in Brazil (n = 88), and non-Amerindian Brazilians, self-identified as white (n = 136), brown (n = 107) or black (n = 118). These cohorts have been described in previous studies from our laboratories, which provide information on institutional review boards and informed consent [2,3].

Allele discrimination at the 7-SNP loci recommended for accurate assignment of *NAT2* haplotypes [101], namely rs1801279, rs1041983 (282C>T), rs1801280, rs1799929 (481C>T), rs1799930, rs1208 (803A>G) and rs1799931 was performed on a Fast 7500 Real-Time System (Applied Biosystems, CA, USA) using validated TaqMan® assays. *NAT2* haplotypes were inferred using PHASE version 2.1 [4,102] and acetylation phenotypes were inferred as described in [101]. Based on the seven genotyped SNPs, individuals were classified as rapid, intermediate and slow acetylators if they possessed zero, one or two slow acetylation *NAT2* haplotypes, namely *NAT2*5*, *NAT2*6*, *NAT2*7* and *NAT2*14*. Inference of NAT2 acetylation phenotype from the 3-SNP and 4-SNP genotype panels was based on the sum of variant alleles at the respective loci: individuals with zero, one or ≥2 variant alleles were classified as rapid, intermediate and slow acetylators, respectively [1,2]. Contingency tables were used to assess the agreement between acetylator phenotypes inferred from the 7-SNPs versus 3- or 4-SNPs. The extent of agreement, expressed as percentage, is used to quantify the accuracy of the 3-SNP and 4-SNP panels to infer the rapid, intermediate and slow acetylator phenotypes. We are aware that these data do not allow strict comparison with the results reported by Hein and Doll [1], since the NAT2 acetylator phenotype was experimentally determined by these authors using sulfamethazine as a substrate, whereas it was inferred from the 7-SNP haplotypes in our study.

We observed 100% agreement between the acetylator phenotypes inferred using either the 7-SNP or the 4-SNP genotype panels in all 6 study groups, namely Mozambicans, Japanese, Guarani–Amerindians, and white, brown and black Brazilians. This observation fully supports Hein and Doll's conclusion that a '4-SNP panel of rs1801279 (191G>A), rs1801280 (341T>C), rs1799930 (590G>A) and rs1799931 (857G>A) infers NAT2 acetylator phenotype with high accuracy' and for economy of scale, is recommended over the 7-SNP genotype panel in non-European populations [1]. Aiming at a further gain in economy of scale, we examined the performance of the 3-SNP genotype panel based on 481C>T,

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590G>A and 857G>A, which Hein and Doll referred to as 'historical' [1], a term that we will adopt from now on. The results of our study (Table 1) indicated that the accuracy of this 3-SNP panel to infer the NAT2 acetylator phenotype ranged from 100% in Guarani and Japanese, to 79.4% in Mozambicans. Among non-Amerindian Brazilians, accuracy declined from white (97.8%) to brown (89.7%) and then to black individuals (80.5%), a trend consistent with the increasing average proportion of African ancestry from white to brown and to black Brazilians [5,6], combined with the poor accuracy of the 3-SNP panel in Mozambicans. It is relevant, in this context, that Mozambique was a major source of enslaved Africans brought to Brazil in the 19th century [7].

The poor performance of the historical 3-SNP panel in Mozambicans led us to examine all other 3-SNP genotype combinations, as predictors of the 7-SNP inferred NAT2 phenotypes in this group (Figure 1). Combined, 191G>A, 341T>C and 590G>A SNPs provided the best results, with correct inference in 98% of the cohort and no instance of extreme phenotype discordance (i.e., slow vs rapid) with the 7-SNP panel (Table 1 & Figure 1). This is a considerable gain in accuracy over the historical 3-SNP panel to predict the acetylator phenotype in Mozambicans. Two other 3-SNP panels outperformed the historical 3-SNP panel in this group: 191G>A, 481C>T and 590G>A (accuracy: 90.2%; no extreme phenotype discordance) and 341T>C, 590G>A and 857G>A (accuracy: 86.3%; extreme phenotype discordance in one individual). Next we tested the accuracy of the 3-SNP panels that outperformed the historical 3-SNP panel in Mozambicans, to predict the NAT2 acetylator phenotype in the other study groups. Table 1 shows that the performance of these panels varied considerably across the cohorts, and no panel provided >90% accuracy in all cohorts. Below, we will highlight the most relevant results for each panel.

The 341T>C, 590G>A and 857G>A panel, reproduced the performance of the historical panel in Japanese and Guarani individuals (100% accuracy), which was anticipated, since the SNPs that distinguish these two panels, namely 341T>C and 481C>T, were in complete linkage disequilibrium in these cohorts. The 341T>C, 590G>A and 857G>A panel outperformed the historical panel in Brazilians, especially in self-reported black (89.8 vs 80.5%) and brown individuals (96.3 vs 89.7%). This is consistent with the relative proportions of African ancestry among the Brazilian groups (see above), combined with the greater accuracy of the 341T>C, 590G>A and 857G>A panel in Mozambicans, relative to the historical panel.

The panels comprising 191G>A, 590G>A and either 341T>C or 481C>T performed equally poorly in Japanese (70.1% accuracy) and in Guarani (62.5%) individuals. This may be explained by 341T>C and 481C>T being in complete linkage disequilibrium, and 191G>A being monomorphic in these two cohorts (data not shown). The rarity of the 191A allele in white Brazilians (0.4%) [2], likely contributed to the reduced accuracy of 3-SNP panels including 191G>A to infer the NAT2 acetylator phenotype in this group. The 191G>A, 341T>C and 590G>A provided the most accurate inference of the NAT2 phenotype in brown (93.5%) and black (90.7%) Brazilians, in line with the considerable African ancestry of these two groups and the high accuracy (98%) of this panel in Mozambicans [5,6].

Collectively, the present analyses verified in Asian (Japanese), African (Mozambican),

Table 1. Agreement between the NAT2 acetylator phenotypes inferred using seven SNPs or different 3-SNP panels in six distinct cohorts.

3-SNP panels	% agreement with 7-SNP (n extreme discordance)†					
	Mozambican (n = 102)	*Japanese (n = 87)*	*Guarani (n = 88)*	*Brazilian*		
				White (n = 136)	*Brown (n = 107)*	*Black (n = 118)*
481C>T, 590G>A, 857G>A‡	79.4 (2)	100 (0)	100 (0)	97.8 (0)	89.7 (1)	80.5 (1)
341T>C, 590G>A, 857G>A	86.3 (1)	100 (0)	100 (0)	99.3 (0)	96.3 (0)	89.8 (0)
191G>A, 341T>C, 590G>A	98.0 (0)	70.1 (4)	62.5 (3)	93.4 (0)	93.5 (0)	90.7 (0)
191G>A, 481C>T, 590G>A	90.2 (0)	70.1 (4)	62.5 (3)	91.9 (0)	86.9 (1)	82.2 (2)

†*Extreme discordance refers to slow vs rapid phenotype misclassification.*
‡*Referred in the text as the historical 3-SNP panel.*

SNP† loci	282	341	481	590	803	SNP loci
191	58.8‡	57.8				**481**
	43.1	98.0§	90.2§			**590**
	42.2	52.0	53.9	68.0		**803**
	34.3	52.9	45.1	51.0	41.2	**857**
282		59.8				**481**
		61.8	59.8			**590**
		48.0	48.0	49.0		**803**
		61.8	59.8	41.2	44.1	**857**
341			76.5			**590**
			48.0	50.0		**803**
			50.0	86.3§	47.1	**857**
481				52.0		**803**
				79.4	49.0	**857**
590					53.9	**857**

Figure 1. Agreement between the NAT2 acetylator phenotype inferred using seven SNPs or 3-SNP panels in Mozambicans (n = 102).
†191G>A rs1801279, 282C>T rs1041983, 341T>C rs1801280, 481C>T rs1799929, 590G>A rs1799930, 803A>G rs1208 and 857G>A rs1799931.
‡Percentage of agreement (accuracy) between the NAT2 acetylator phenotype inferred using seven SNPs or 3-SNP panels.
§The panels which were tested in the other study cohorts, results are shown in Table 1.

Native American (Guarani) and admixed Brazilian cohorts the conclusion advanced by Hein and Doll [1], that a 4-SNP panel of rs1801279 (191G>A), rs1801280 (341T>C), rs1799930 (590G>A) and rs1799931 (857G>A) may be used, with economy of scale, to infer with 100% accuracy the NAT2 acetylator phenotype. Two independent studies, have recently shown that neither the tag-SNP rs1495741 nor 2-SNP panels provide sufficiently accurate prediction of NAT2 phenotypes for global health studies [2,8]. The present data extend this conclusion to 3-SNP panels. However, both the historical and the 341T>C, 590G>A and 857G>A 3-SNP panels predict the NAT2 acetylator phenotype with 100% accuracy in Japanese, and 98–99% accuracy in white Brazilians. Of note, the high accuracy (98%) provided by the 191G>A, 341T>C and 590G>A 3-SNP panel in Mozambicans may prove sufficient for specific clinical or research applications in this, and possibly, other African groups. Finally, the fact that no 3-SNP panel inferred with >91% accuracy the NAT2 acetylator phenotypes in brown and black Brazilians, highlights the implications of population admixture, heterogeneity and diversity in pharmacogenomics investigation and adoption of pharmacogenomics-informed drug prescription in clinical practice [9].

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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