Faculdade de Medicina de São José do Rio Preto
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Malária vivax no Estado do Pará: influência de polimorfismos nos genes *TNFA*, *IFNG* e *IL10* associados à resposta imune humoral e ancestralidade genômica.

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Orientador: Prof. Dr. Ricardo Luiz Dantas Machado

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ADRIANA ANTÔNIA DA CRUZ FURINI

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SUMÁRIO

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“Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos”.

(Isaac Newton)

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LISTA DE ABREVIATURAS E SÍMBOLOS

ADCI - Inibição Celular Dependente de Anticorpo

AIMs - Marcadores Informativos de Ancestralidade

Anova - Análise de variância

APC - Células apresentadoras de antígenos

B7-1 - Ligante da molécula co-estimuladora CD28 expresso na superfície de linfócitos B e macrófagos/monócitos (CD80)

B7-2 - Ligante da molécula co-estimuladora CD28 expresso na superfície de linfócitos B e macrófagos/monócitos (CD86)

BCMA - Antígeno de maturação de linfócito B

Blys - Estimulante de linfócitos B

BR3 - Blys receptor 3

CD28 - Receptor de Linfócitos T (Cluster de diferenciação 28)

CD28 - Gene *CD28*

CD40 - Receptor CD40 (Cluster de diferenciação 40)

CD40L - Ligante de CD40

CD80 - Ligante da molécula co-estimuladora CD28

CD86 - Ligante da molécula co-estimuladora CD28

CSP - Proteína circumesporozoítica

CTLA-4 - Antigénio 4 dos linfócitos T citotóxicos

ºC - Grau Celsius

DNA - Ácido desoxirribonucleico

dNTP - Desoxirribonucleótidos trifosfato

Duffy - Antígeno de superfície das hemácias

ELISA - Ensaio imunoenzimático

FcγRIIa - Receptor de fração constante de anticorpos (CD32)

G6PD - Glicose 6-fosfato desidrogenase

HLA - Antígeno leucocitário humano

HbS - Hemoglobina S

HWE - Equilíbrio de Hardy-Weinberg

ICB2-5 - Proteína N-terminal da MSP-1 do *Plasmodium vivax*

ICOS - Proteína imunológica indutível co-estimuladora

ICOSL - Ligante da molécula ICOS

IgA - Imunoglobulina A

IgE - Imunoglobulina E

IgG - Imunoglobulina G

IgG2 - Imunoglobulina G da subclasse 2

IgG3 - Imunoglobulina G da subclasse 3

IgG4 - Imunoglobulina G da subclasse 4

IgM - Imunoglobulina M

IL-1 - Interleucina 1

IL-2 - Interleucina 2

IL-3 - Interleucina 3

IL-4 - Interleucina 4

IL-6 - Interleucina 6

IL-8 - Interleucina 8

IL-10 - Interleucina 10

IL10 - Gene da Interleucina 10

IL-13 - Interleucina 13

INDELS - Inserção/deleção

INFG - Gene do Interferon Gama

INFγ - Citocina Interferon Gama

IR - Índice de Reatividade

LTCD4 - Linfócito TCD4

LTCD8 - Linfócito TCD8

Linfócitos Tγδ - Linfócitos T gama-delta

LB - Linfócitos B

MgCl2 - Cloreto de magnésio

µg - Micrograma

MHC - Complexo Maior de Histocompatibilidade

µL - Microlitro

nM - Nano molar

MSP-1 - Proteína 1 da superfície de merozoíto

NFkB - Via NFK beta

OD - Densidade ótica

pB - Pares de bases

PBS - Tampão fosfato salino

PCR-ASO - PCR Alelo Específica

Pg - Picograma

pH - Potencial Hidrogeniônico

pmol - Picomol

P. vivax - *Plasmodium vivax*

PvAMA-1 - Antígeno de membrana apical-1 do *Plasmodium vivax*

PvDBP - Proteína do Plasmodium vivax, ligante do Duffy

Pv MSP-1_{19} - Proteína da superfície de merozoíto do Plasmodium vivax

RI - Reactivity Index

RFLP - Polimorfismo do tamanho do fragmento de restrição

SNP - Polimorfismo de nucleotídeo único

TACI - Receptor do linfócito B interagente ligante ciclofilina

TCLE - Termo de consentimento livre e esclarecido

TCR - Receptor de células T

TGFBeta - Fator de Transformação de crescimento (Citocina)

Th1 - Linfócitos T auxiliares (1)

Th2 - Linfócitos T auxiliares (2)

TNF-α - Fator de necrose tumoral alfa (Citocina)

TNFA - Gene do fator de necrose tumoral alfa

TNFR1 - Receptor do Fator de Necrose Tumoral Alfa

VK247 **-** Variante 247 do *Plasmodium vivax*

RESUMO

Introdução: A malária é uma das maiores causas de morbidade e mortalidade em países tropicais e subtropicais. **Objetivos:** Avaliar a influência da ancestralidade genética na distribuição de polimorfismos em genes envolvidos na resposta imune e os níveis de anticorpos contra proteínas expressas no estágio de merozoíto do *Plasmodium vivax*. **Material e Métodos:** Foram avaliados 90 indivíduos com malária vivax e 51 não infectados de Goianésia do Pará, região Norte do Brasil. Nove polimorfismos de nucleotídeo único (SNPs) distribuídos nos genes: *TNFA, INFG* e *IL10* foram genotipados por PCR-ASO ou PCR-RFLP. A ancestralidade genômica para os três grupos étnicos (africana, europeia e ameríndia) foi categorizada com a utilização de 48 INDELs. As respostas de anticorpos específicos contra as proteínas C-terminal ($MSP\text{-}1_{19}$) da MSP-1, da DBP e da AMA-1 do *P. vivax* foram determinadas por ELISA. **Resultados**: Não houveram diferenças nas proporções de ancestralidade na maioria dos SNPs investigados, apenas para o alelo TNF-308A e a ancestralidade europeia. Nenhuma associação significativa foi observada entre as frequências alélicas e genotípicas dos SNPs entre os grupos investigados. Não foi encontrada diferença significativa nos níveis de anticorpos IgG em relação aos polimorfismos estudados. **Conclusões:** Esses resultados ressaltam que os polimorfismos nos genes *TNFA, INFG* e *IL10* não influenciam na resposta imune anti-merozoítos do *P. vivax*. Discutimos o perfil imunogenético envolvido na resposta imune humoral na malária vivax em região endêmica da Amazônia brasileira.

Palavras-Chave: Anticorpos*. IFNG. IL10*. *Plasmodium vivax. TNFA*

ABSTRACT

Introduction: Malaria is one of the mayor cause of morbidity and mortality in tropical and subtropical countries. **Objectives:** To evaluate the influence of genetic ancestry in the distribution of polymorphisms in genes involved in the immune response and antibody levels against proteins expressed in the merozoite stage of *Plasmodium vivax*. **Material and Methods:** To evaluated 90 patients with *vivax malaria* and 51 non-infected patients from Goianésia do Pará, northern Brazil. Nine single nucleotide polymorphisms (SNPs) in the genes: TNFA, IL-10 INFG were genotyped by PCR-ASO or RFLP-PCR. The genetic ancestry for three ethnic groups (African, European and American Indian) were categorized using 48 INDELs. The responses of specific antibodies against the C-terminal proteins (MSP-119) MSP-1, BPD and AMA-1 of *P. vivax* were determined by ELISA. **Results:** There were no differences in ancestry proportions in most SNPs investigated only for TNF-308A allele and European ancestry. No significant association was observed between the allele and genotype frequencies of the SNPs between the groups investigated. There was no significant difference in the levels of IgG antibodies to the studied polymorphisms. **Conclusions**: These results indicated that the polymorphisms in the *TNFA, INFG* e *IL10* genes can not influence the anti-merozoites immune response of *P. vivax*. We discussed the immunogenetic profile involved in the humoral immune response in malaria vivax in an endemic area of the Brazilian Amazon.

Keywords: Antibodies. *IFNG*. *IL10*. *Plasmodium vivax*.*TNFA*.

INTRODUÇÃO

1. INTRODUÇÃO

1.1 Considerações gerais: epidemiologia, transmissão e agentes etiológicos.

Apesar dos progressos nas estratégias de controle da malária, a doença ainda é uma das maiores causas de morbidade e mortalidade em muitos países tropicais e subtropicais. [1,2,3] Cento e quatro países são endêmicos com 207 milhões de casos clínicos por ano e aproximadamente 627.000 mil mortes.[3] Nas Américas, três países concentram 76% dos casos de malária, sendo o Brasil responsável por 52% dos casos. [4]

Os perfis de transmissão da doença no Brasil são diferentes e observados em três ambientes distintos. Na Amazônia e na Mata Atlântica, ambos com uma predominância de casos autóctones, e em outras regiões, com casos importados de recentes viagens a áreas endêmicas de malária no país, ou em outros da América Central e do Sul, países africanos ou asiáticos [5,6].

A malária é uma doença protozoária na qual a infecção ocorre pela inoculação de esporozoítos de *Plasmodium* por meio da picada de fêmeas do mosquito do gênero *Anopheles.* [4,6] O ciclo da doença é heteroxênico, com fase sexuada no vetor e assexuada no homem. No vertebrado, ocorre esquizogonia hepática e eritrocitária. [7,8,9] Cinco espécies de *Plasmodium* são responsáveis pela etiologia humana da malária: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* e, recentemente,

o *Plasmodium knowlesi* foi detectado na Malásia.[3,10] O *P. falciparum* está associado aos maiores índices de morbimortalidade, enquanto que o *P. vivax* é amplamente disseminado pelo mundo.[3,11,12] No Brasil, o *P. vivax* tem sido responsável por aproximadamente 85% dos casos. [3,4]

1.2 Resposta imune no paciente com malária

1.2.1 Participação de citocinas e do receptor CD28 na resposta imune a malária.

Mecanismos inatos, humorais e celulares são envolvidos na resposta imune da malária, com a participação de células, citocinas, receptores e anticorpos, que podem eliminar o agente etiológico ou acarretar em complicações imunopatológicas.[7,12,13] Os linfócitos $TCD4^+$ (auxiliares) participam das respostas imunes celulares e humorais por meio da ativação por citocinas pro e anti-inflamatórias. Essas células são fundamentais para ativação de linfócitos B (LB) por citocinas anti-inflamatórias que resultam na diferenciação em plasmócitos e secreção de anticorpos. [12,14]

Os receptores de antígenos dos linfócitos T (LT-TCR), e os co-receptores CD4 ou CD8 ligam-se ao complexo maior de histocompatibilidade (MHC) de células apresentadoras de antígenos (APCs), para ativação dos linfócitos T (LT) na resposta imune celular.[15,16,17] No entanto, essa ligação não determina a expansão clonal dos LT, que requer um segundo sinal co-estimulatório que é emitido pela mesma APC, por meio de glicoproteínas de membrana denominadas de B7.1 ou CD80 e B7.2 ou CD86.[16] O receptor

dessas moléculas nas células T é o CD28, expresso constitutivamente na superfície dessas células.[15] A ligação do CD28 com seus ligantes (CD80 ou CD86) potencializa a transcrição e produção da interleucina-2 (IL-2), que resulta em proliferação e expansão clonal das células T[15,16] e liberação de outras citocinas. O significado da coestimulação via CD28 no desenvolvimento da imunidade depende do agente etiológico, como reportado em infecções por *Salmonella enterica* [18] e *Trypanosoma cruzi* [19], mas com pouca, ou nenhuma função na imunidade contra *Toxoplasma gondii* [20].

Em relação à malária, Taylor-Robinson e Smith (1994) [21] reportaram que o tratamento de camundongos infectados pelo *Plasmodium chabaudi* com anticorpos monoclonais anti-CD86 impediu o clareamento da parasitemia, sugerindo uma possível função da via CD86/CD28 no controle da malária crônica. Por outro lado, Kemp e colaboradores (2002) [22] avaliaram a expressão de IFN-γ e IL-4 por LTCD28$^+$ e LTCD28$^-$ em crianças africanas com malária falciparum, e verificaram que os níveis de IFN-γ produzidos pelas LTCD28$^-$ foram menores. Elias e colaboradores [16], no ano de 2005, avaliando o papel do CD28 em modelo murino, encontraram que após uma semana de infecção a expressão de IFN-γ foi 50% menor nos LTCD28$^-$.

Assim como as células e receptores possuem um papel fundamental na resposta imune ao *Plasmodium*, o balanço entre as citocinas pró (Th1 - celulares) e antiinflamatórias (Th2 - humorais) é crucial para o prognóstico na malária. [7,13, 14, 23-25] A superprodução e persistência desses mediadores podem levar a imunopatologia, com gravidade e óbito [13,14,24,26,27], mas por outro lado,

pequenos níveis não são suficientes para inibir o crescimento do parasito. (9,14,26)

O fator de necrose tumoral alfa (TNF-α) é uma citocina pró-inflamatória que participa no recrutamento e ativação de monócitos, macrófagos e neutrófilos para o sitio da infecção,(28,29), na modulação positiva para resposta imune humoral de IgG total (30), como fator de crescimento autócrino para os LB. (31) Na patogênese da febre atua em conjunto com a interleucina-1 (IL-1) para ativação de células hipotalâmicas, além de participar da negativação parasitária tanto *in vivo* com *in vitro.*(26,32) Níveis elevados TNF-α estão relacionados ao paroxismo malárico(33) , malária grave(31) e malária cerebral(9) .

Respostas acentudas do interferon gama (IFN-γ) são reportadas no controle de infecções agudas por *Plasmodium berghei*, *Plasmodium yoelii*, e *P.chabaudi* em modelos murinos e para o *P.falciparum* na malária humana. (25) Essa citocina pró-inflamatória é produzida por LTCD4, LTCD8, linfócitos T γδ e *natural kille* (34) e contribui para o controle da infecção na fases hepática e eritrocítica (33,34) com ativação de macrófagos e outras APCs. Corrobora na modulação negativa para a resposta imunológica do tipo anti-inflamatória (Th2), contribui para o processo de homeostase e pode aumentar a produção de IgG2.(35) Por outro lado, o balanço de citocinas é obtido pela modulação negativa das anti-inflamatórias IL-10 e TGF-beta na resposta do tipo pró-inflamatória (IL-1, IL-6, IL-8, IL-12, IFN-γ e TNF-α) do tipo Th1. (25,26,34,36-38)

O excesso de resposta TH1, na incapacidade de produção de IL-10 e TGF-beta, pode acarretar em inflamação excessiva e dano tecidual na malária.(14) A IL-10 também sinergiza a produção dos anticorpos IgG, IgA e

IgM, induzidas por IL-4.[31,38] Na malária altos níveis de IL-10 estão relacionados ao clareamento da parasitemia.[14, 37]

1.2. 2 Polimorfismos no genes *CD28*, *TNFA*, *IFNG* E *IL10*

A susceptibilidade e resistência para malária podem estar relacionadas à seleção natural, fatores genéticos do hospedeiro e do agente, idade, etnia, e esses por sua vez, envolvidos na resposta imunológica, sintomas e níveis de parasitemia.[39-41] Corroboram também as situações epidemiológicas, ambientais, geográficas e de tempo de moradia em regiões endêmicas.[2,41] Polimorfismos em genes de citocinas tem sido [13,27] associado com níveis circulantes dessas proteínas e de anticorpos [39,42] na malária para evolução clínica e prognóstico.[27] Dessa maneira polimorfismos de nucleotídeo único (SNPs) podem influenciar no desenvolvimento de vacinas e de novas alternativas terapêuticas para malária.[40,43]

Um SNP na posição +17T/C (rs3116496), situado no íntron 3 do receptor *CD28* localiza-se próximo a um sítio de recomposição que pode interferir na eficiência desse receptor. Associações significativas foram descritas entre esse SNP com diabetes de tipo 1 [44] e artrite reumatóide. [45] Na malária o papel deste polimorfismo foi descrito por Cassiano e colaboradores [46] quanto a presença do alelo T com níveis mais baixos de IgG1 específica para a proteína ICB2-5. Na posição -372G/A (rs35593994) no gene *CD28* as duas variantes alélicas foram caracterizadas na população australiana, porém sem associação com a esclerose múltipla. [47] Esta variação alélica também foi descrita em amostragem da população brasileira, mas sem associação com pênfigo foliáceo.[48]

O gene *IL10* é localizado no cromossomo 1q3-q32 e apresenta pelo menos 27 sítios polimórficos. [27,37, 49] Na região promotora do gene, SNPs tem sido associados à produção de citocinas [37] e níveis de anticorpos[39,42,50] na malária. Os haplótipos IL10 -1082/-819/-592 GCC, ACC e ATA são associados respectivamente à alta, intermediária e baixa atividade de transcrição da citocina. [27,37,51]

O IFN-γ é codificado pelo gene situado no cromossomo 12q24.1, que consiste de 4 exons e 3 introns. [52-54] Polimorfismos no gene do *IFNG* tem sido associados com tuberculose [51,55], dermatite[56] , mas não com artresia biliar. [53] Na posição -183G/T (rs2069709), o alelo T foi associado ao aumento da atividade de transcrição [52,53] malária cerebral na África Ocidental [52], Hepatite B na China. [57] O SNP +874 A/T (rs2430561) está localizado no intron 1 do gene do *IFNG* e influencia a expressão do RNAm e secreção da proteína. [54] O alelo T é associado com elevada produção de citocina. [13,56]

O gene do *TNFA* é situado no cromossomo 6p21.3, em uma região altamente polimórfica [51,58] entre os genes do HLA de classe I e classe II. [43] O SNP na posição +308G/A (rs1800629) tem sido amplamente estudado na malária. [59-62] Apesar de maior distribuição do alelo ancestral, com aproximadamente 87% [63, 64], o alelo A é descrito para aumento nos níveis da proteína, porém sem efeito aparente na susceptibilidade ao *P. vivax* [26,65] ou com resistência ao *P. falciparum* . [62] Os alelos T [-1031T/C (rs1799964)] e G (-308G/A) foram associados com episódios de malária não complicada [29] em Burkina Faso. Para os SNPs -238G/A (rs361525) e para o -308G/A o alelo A foi associado com redução da parasitemia na malária. [29,66]

1.2.3 Polimorfismos em genes de citocinas e ancestralidade

No Brasil, a heterogenicidade tri-híbrida da população é originária da migração de nativo americanos (asiáticos) para o continente por meio do Estreito de Bering [67], seguida da colonização Europeia no Nordeste brasileiro a partir de 1530, e fluxo migratório de escravos africanos. [68,69] A miscigenação populacional pode ser uma causa para resultados não totalmente esclarecidos ou contraditórios na distribuição de alelos e genótipos envolvidos na transcrição, expressão do gene e produção de citocinas. [69-71]

A susceptibilidade à malária ou fenótipos tem sido avaliada por estudos de associação, do tipo caso e controle [41,43,72], com etnia auto declarada ou indicadores de aparência física, nos quais existe o risco de se encontrar associações espúrias. Esse fato decorre da estratificação populacional, ou em populações miscigenadas com diferentes frações de ancestralidade.[70,71] Dessa maneira, a análise de ancestralidade por meio de marcadores informativos de ancestralidade (MIAs) do tipo inserção e deleção (INDEL), pode contribuir para eliminar a possibilidade das associações espúrias. Entretanto, poucos estudos demonstram as diferenças étnicas na distribuição de SNPs baseado em uso de MIAs [17, 71,73], e para a ancestralidade genética nativo americana esses dados são ainda mais escassos. [74,75]

1.2.4 Polimorfismos em genes de citocinas e produção de anticorpos na malária

Epítopos imunogênicos da superfície do parasito, tanto de proteínas do esporozoíto e merozoíto têm sido amplamente estudados como potenciais alvos para formulação de vacinas. Em geral o principal marcador de proteção são anticorpos anti-merozoítos [7] descritos em estudos conduzidos na Amazônia brasileira. [76,77,78,79] As proteínas do merozoíto mais estudadas são as que participam do processo de invasão dos eritrócitos, como a $MSP1_{19}$, do inglês *Merozoite Surface Protein-1* [80,81] a DBP, do inglês *Duffy Binding Protein* [82,83] e AMA-1, do inglês *Apical Membrane Antigen-1.* [78,84,85]

A imunidade humoral para o *P. vivax* é descrita para ser mais rápida do que para o *P. falciparum*[23,86], entretanto vários anos de exposição contínua em áreas endêmicas é necessária para a situação de premunição e redução do risco de malária clínica, com baixas parasitemias e altos níveis de anticorpos anti-merozoítos. [7,23,39]

A resposta imune humoral na malária vivax é amplamente descrita para estar associada hemoglobinopatias, traço falciforme (HbS), deficiência de G6PD, [40,41] variabilidade genética do HLA, [78] antígeno Duffy[41,87] variantes da proteína CSP de *P.vivax* [23,24,88] e mais recente por polimorfismos em genes de citocinas e de moléculas co-estimulatórias da resposta imune. [13,27,43,46,72,79,85]

Polimorfismos em genes de citocinas tem sido [13,27] associados com níveis e classes de anticorpos [39,42] na malária. Em estudos (Tanzânia) com *P.falciparum*, para os SNPs no gene da *IL-10* (-592) e (-1082), o alelo A foi associado a baixos níveis de IgE e IgG4, [50] o genótipo AA de IL-10-1082 com altos níveis de anticorpos para AMA-1 e MSP2-3DT em mães e recém-

nascidos.[39] O alelo A do *TNFA* nas posições -308 [50] e -238 [29] com altos níveis de anticorpos IgG anti *P. falciparum.* No Brasil, não foram descritas associação de SNPs (-590C/T, IL4 -33C/T e o VNTR) no gene da *IL4* com a parasitemia ou com níveis de anticorpos contra a PvAMA-1 [85] em indivíduos maláricos do município de Goianésia do Pará, e também entre anticorpos contra esporozoítos e merozoítos de *P. vivax* com SNPs no genes *CD40* e *BLyS* numa população de Macapá no Estado do Amapá. [72] Por outro lado, indivíduos infectados naturalmente por *P. vivax* em Goianésia do Pará, os SNPs nos genes do *BLYS* (–871C/T) foram associados com a frequência de respostas IgG para PvAMA-1 e PvMSP-119, no gene do *CD40* (-1C/T) para IgG contra PvDBP e no gene do *CD86* (+ 1057G/A) para IgG contra PvMSP-$1_{19.}$ [79]

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar a influência de polimorfismos nos genes *CD28*, *INFG*, *TNFA* e *IL-10* na resposta imune humoral na malária vivax.

2.2 Objetivos Específicos

a) Determinar as frequências alélicas e genotípicas de variantes nos *genes INFG TNFA* e *IL-10* em indivíduos com malária vivax e indivíduos não infectados.
b) Avaliar a frequência de polimorfismos nos genes *TNFA*, *IFNG* e *IL10* em uma amostra da população brasileira, relacionando suas distribuições às frações de ancestralidade genética determinada com o auxílio de Marcadores Informativos de Ancestralidade.
c) Estabelecer possíveis associações entre os polimorfismos e proteção na malária vivax.
d) Identificar possíveis associações entre os polimorfismos de genes de citocinas e haplótipos com níveis de anticorpos para PvDBP, Pv-AMA-1 e Pv-MSP-1-$_{19}$.

ARTIGOS CIENTÍFICOS

ARTIGOS CIENTÍFICOS

Esse trabalho é composto de dois artigos originais.

Artigo 1

Título: *TNF-alpha*, *IFN-gamma* and *IL10* cytokine SNPs: Comparison of polymorphisms by genomic ancestry in an admixed population.

Autores: Adriana Antônia da Cruz Furini, Gustavo Capatti Cassiano, Marcela Petrolini Capobianco, Sidney Emanuel dos Santos, Ricardo Luiz Dantas Machado.

Periódico: Mediators of Inflammation (artigo aceito para publicação). Fator de impacto: 3,418

Artigo 2

Título: Cytokine gene polymorphisms are not associated with anti-Pv-DBP, Pv-AMA-1 or Pv-MSP-1_{19} IgG antibody levels in a malaria-endemic area of the Brazilian Amazon.

Autores: Adriana Antônia da Cruz Furini, Marcela Petrolini Capobianco, Luciane Moreno Storti-Mello, Maristela Gomes da Cunha, Gustavo Capatti Cassiano, Ricardo Luiz Dantas Machado.

Periódico: Malaria Journal (artigo publicado). Fator de impacto: 3,079

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Best regards,

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Frequency of *TNFA*, *INFG* and *IL10* gene polymorphisms and their association with malaria vivax and genomic ancestry

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BACKGROUND

Polymorphisms in cytokine genes can alter the production of these proteins and consequently affect the immune response. The tri-hybrid heterogeneity of the Brazilian population is characterized as a condition for the use of ancestry informative markers. The objective of this study was to evaluate the frequency of *TNFA*, *INFG* and *IL10* gene polymorphisms and their association with malaria vivax and genomic ancestry

. Samples from 90 vivax malaria-infected individuals and 51 non-infected individuals from northern Brazil were evaluated. Six single nucleotide polymorphisms (SNPs) in *TNF-alpha, IFN-gamma* and *IL10* genes were genotyped using allele-specific oligonucleotide polymerase chain reaction or PCR/RFLP. The genomic ancestry of the individuals was classified using 48 insertion/deletion polymorphism biallelic markers. There were no differences in the proportions of African, European and Native American ancestry between men and women. No significant association was observed for the allele and genotype frequencies of the 6 SNPs between malaria-infected and non-infected individuals. However, the frequency of individuals carrying the TNF-308A allele decreased progressively with the increasing proportion of European ancestry. No genotypic marker appeared in only one ethnicity, and there was no allelic or genotypic association with susceptibility or resistance to vivax malaria. Understanding the genomic mechanisms by which ancestry influences this association is critical and requires further study.

1. Introduction

With the completion of the Human Genome Project and the ease of identifying variations in DNA using currently available tools, several studies on genetic associations have evaluated the genetic bases of certain traits (e.g., the susceptibility to or different clinical manifestations of various types of diseases, including diabetes, cancer and hypertension, as well as autoimmune, infectious parasitic and cardiac diseases) [1, 2, 3, 4, 5, 6]. These association studies are based on comparisons of the allele frequencies of candidate genes between a group of people who have the disease or the outcome of interest and an unaffected group [7,8].

Malaria is one of the most studied infectious diseases. It is the primary parasitic disease worldwide and is responsible for approximately 214 million cases annually, resulting in more than 438, 000 deaths [9]. Currently, it is widely accepted that genetic factors of the human host contribute to the infection and different clinical manifestations of the disease [10, 11, 12]. The observed genetic variants associated with malaria include those present in erythrocytes, which play an essential role as host cells during the asexual life cycle of the parasite [13,14,15]. Moreover, polymorphisms in cytokine genes can alter the production of these proteins and consequently affect the inflammatory response to malaria [16,17,18], and they may be associated with susceptibility to or progression of the disease [17, 19].

The prognosis of *Plasmodium* infection depends on the balance between pro- and anti-inflammatory cytokines [20, 21, 22, 23] . IFN-γ, TNF-α, IL-6, IL-12, IL-1β and IL-8 are reported at higher levels in individuals infected with *Plasmodium* than in controls or in individuals with severe malaria [21, 24, 25]. However, contradictory results have also been observed, with lower levels of these cytokines reported in infected patients [25,26].

TNF-α participates in tumorigenesis, apoptosis, immune cell activation, hyperthermia [18,22] and parasitemia reduction [27,28]. However, it can play different, concentration-dependent roles in malaria, ranging from protection against the destructive activity of infection on the vascular and brain endothelium to changes in blood glucose levels [29,30]. SNPs in this gene have the potential to alter transcription factors, influencing the circulating levels of the cytokine [16]. The A (-308) and C (-1031) alleles have been associated with circulating levels of the cytokine and with clinical symptoms but not with susceptibility [27], whereas the G allele (-308) has been associated with increased susceptibility to malaria vivax [19]. Other alleles at positions -1031T, -863C, -857T, -308G, and -238G have been associated with an increased risk of developing cerebral malaria in patients in Myanmar [31].

IFNG acts as a regulator of antigen presentation, proliferation and differentiation in lymphocyte populations and plays a modulatory role in the immune response mediated by anti-inflammatory cytokines [32], such as IL-10. This Th2-type cytokine has a negative immunoregulatory effect [33,34] on IL-1, IL-6, IL-8, IL-12, IFN-γ and TNF-α [17,27] that is essential for maintaining homeostasis and limiting tissue damage by infectious agents

[34]. The production of the IgG, IgA and IgM isotypes induced by IL-4 is synergistic [35] However, high levels can contribute to the maintenance of the parasite in the host and can be related to cerebral malaria and high levels of parasitemia [20,2124].

However, certain aspects of these observed associations have proven irreproducible in subsequent studies performed in different populations [36,37,38], with contradictory results for different SNP associations with susceptibility to different *Plasmodium* species and levels of circulating cytokines and antibodies.. There are many reasons for the lack of consistency in these results, but discrepancies are often due to population stratification, which can occur in populations with different allele frequencies between and within subgroups [8]. If the population subgroups are represented in different proportions between individuals of the case and control groups, then spurious associations may be observed; thus, ancestry informative markers (AIMs) have been employed in an attempt to avoid the population stratification problem [39,40].

This consideration is particularly important in studies involving admixed populations, as is the case in the Brazilian population due to crosses involving primarily Europeans, Africans and Native Americans. Previous studies employing AIMs in Brazil demonstrated that the allele distributions in genes involved in pharmacokinetics [41,42] or in the co-stimulation of B and T lymphocytes [43] were affected by the proportions of genetic ancestry. The frequencies of several cytokine gene alleles vary significantly among some ethnic groups and geographic populations. Moreover, the lack of data on Native Americans in the Brazilian population motivated us to investigate the frequency of polymorphisms in *TNFA*, *INFG* and *IL10* genes in people living in a malaria endemic area of the Brazilian Amazon e their possible association with malaria vivax and genomic ancestry.

2. Materials and Methods

2.1 Sample

The sample used in this study was from the municipality of Goianésia, Pará (03° 50’ 33” S; 49° 05’ 49” W), Brazil, which is a malaria-endemic area in the Brazilian Amazon. The sample was a subset of the individuals analyzed in Cassiano et al., 2015 [43]. A total of

141 unrelated individuals older than 14 years were recruited at the Goianésia malaria diagnosis center. Of these individuals, 90 were diagnosed with vivax malaria by microscopy, and infection was subsequently confirmed using nested-PCR; no infections by any human malaria species were observed in the remaining 51 individuals. All participants or guardians signed the consent form, and the project was approved by the Goianésia do Pará health authorities and by the Research Ethics Committee (CAAE 01774812.2.0000.5415) of the College of Medicine of São José do Rio Preto (Faculdade de Medicina de São José do Rio Preto).

2.2 Genotyping

DNA was extracted using an Easy-DNA™ extraction/purification kit (Invitrogen, CA, USA).

***2.2.1 TNFA* genotyping: Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP).**

The following oligonucleotides were used for the -308 G>A position (*rs1800629*): forward 5'- GAG GCA ATA GGT TTT GAG GGC CAT -3' and reverse 5'- GGG ACA CAC AAG CAT CAAG -3'. A quantity of 2.1 μl of DNA was used in 2.5 μl of 1x buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 2.5 μl of glycerol, 1.5 mM $MgCl_2$, 0.2 μM of each dNTP; 1.5 μl of each primer, and 0.1 μl of Taq Platinum (0.5 U) (Invitrogen, São, Paulo, Brazil). The amplification process consisted of an initial denaturation step of 94°C for 5 min and 35 denaturing cycles (94°C for 30 s, 59°C for 30 s, and 72°C for 1 min), which was followed by a final extension at 72°C for 5 min. The PCR products were visualized on a 2% agarose gel stained with 2.5% GelRed™ (Biotium, Hayward, USA). The PCR products at 147 bp were digested with *Nco*I (Fermentas, Vilnius, Lithuania) restriction endonuclease for 15 minutes at 37°C to identify the genotypes [28]. The digestion products were stained with 2.5% GelRed™ (Biotium, Hayward, USA) and viewed on a 12.5% polyacrylamide gel after ethidium bromide staining..The resulting

fragment for the A/A genotype was 147 bp, while the fragments for the G/G genotypes were 126 and 121 bp, and those for the G/A genotypes were 147, 126 and 21 bp [44]

The following oligonucleotides were used for the *TNFA*-1031T>C position (*rs 1799964*): forward 5'-TAT GTG ATG GAC TCA CCA GGT -3' and reverse 5'- CCT CTA CAT GGC CCT GTC TT -3'. Genomic DNA (3.0 μl) was amplified with 0.1 μl of Taq Platinum (0.5 U) (Invitrogen, São, Paulo, Brazil), 1.5 mM $MgCl_2$, 0.2 μM of each dNTP and 1.5 μl of each primer. Polymerase chain reactions were run for 35 cycles: 5 min at 94°C, 30 s at 57°C, and 1 min at 72°C, followed by a final extension at 72°C for 5 min. These oligonucleotides generated a 251-bp fragment visualized on a 2% agarose gel stained with 2.5% GelRed™ (Biotium, Hayward, USA). The product (10 μl)
was digested with 10,5 μL of *Bbs*I (Fermentas, Vilnius, Lituânia) –at 37ºC for 12 h, subjected to electrophoresis in a 12.5% polyacrylamide gel after ethidium bromide staining, resulting in 251 and 13 bp fragments for the TT genotype; 251, 180, 71 and 13 bp fragments for the T/C genotype; and 180, 71 and 13 bp fragments for the CC genotype [44].

The PCR and RFLP reactions for the *TNFA*-238G>A position *(rs361525)* were standardized according to the protocols of Hedayati et al., 2012 [45]. The following oligonucleotides were used: forward 5'-ATC TGG AGG AAG CGG TAG TG -3' and reverse 5'- AGA AGA CCC CCC TCG GAA CC -3'. Briefly, amplification was performed in a final volume of 25 μl containing 1.0 μl of total extracted DNA, 0.1 μl of Taq Platinum (0.5 U) (Invitrogen, São, Paulo, Brazil), 1.5 mM $MgCl_2$, 0.2 μM of each dNTP, and 1.0 μl of each primer. The amplification reactions were performed under the following conditions: initial denaturation for 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C; and a final extension of 5 min at 72°C, which generated a 153-bp fragment that was visualized on a 2% agarose gel stained with 2.5% GelRed™ (Biotium, Hayward, USA). A total of 10 μl of the PCR product was subjected to restriction enzyme digestion with MspI (Thermo Scientific) using 10.5 μl of the required enzyme at 37°C for 15 min. The genotypes were identified as AA for the 156-bp fragment, GG for the 133-bp fragment and G/A for 153- and 133-bp fragments in a 2% agarose gel stained with 2.5% GelRed™ (Biotium, Hayward, USA)

2.2.2 IL10 genotyping*:* Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP).

For the *IL10* SNPs at the -592C>A *(rs 1800872)* and -819 C>T positions *(1800871)*, the reactions were standardized in-house with the following oligonucleotides: forward 5'-GGG TGA GGA AAC CAA ATT CEC -3' and reverse 5'- GAG GGG GTG GGC TAA ATA TC -3'. The 25 μl PCR mixture contained 1.0 μl of total extracted DNA, 0.1 μl of Taq Platinum (0.5 U) (Invitrogen, São, Paulo, Brazil), 1.5 mM $MgCl_2$, 0.2 μM of each dNTP, 1.2 μl of each primer, and 2.5 μl glycerol. The cycling conditions were as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. These oligonucleotides generated a 361-bp fragment. The PCR products were digested overnight at 37°C with 0.5 μl of *RseI* (Fermentas, Vilnius, Lithuania), and in another reaction, 10 μl of the PCR product for the IL10-819C>T SNP was digested with 0.5 μl of the enzyme *RsaI* (Invitrogen, CA, EUA) for the IL10-592C>A SNP. After digestion, the fragments generated at the -592C>A position were 240, 77, 36 and 8-bp for the AA genotype; 317, 36 and 8bp for the CC genotype; and 317, 240, 77, 36 and 8-bp for the CA genotype. At the -819 position, the TT, CC and TC genotypes were identified with 270 and 91bp; 217, 91 and 53bp; and 270, 217, 91 and 53bp bands, respectively. A 2% agarose gel stained with 2.5% GelRed™ (Biotium, Hayward, USA) was used.

2.2.3 IFNG genotyping*:* ASO-PCR

The polymorphism at the +874A>T position in the *IFNG* gene (*rs 2430561*) was identified using allele-specific oligonucleotide-polymerase chain reaction (ASO-PCR [21] . The following oligonucleotides were used: IFNG (+874) CP: 5'-TCA ACA CTG ATA

AAG CTC AC-3 ', IFNG (+874) T: 5'-TTC TTA CAA CAC AAA ATCAAA TCT -3 ', or IFNG (+874) A: 5'-TTC TTA CAA CAC AAA ATC AAA ATC-3'.

These oligonucleotides resulted in a 262-bp fragment after changing the annealing conditions from 56°C for 40 s to 53°C for 1 min, modified from Medina et al., 2011 [30]. The amplified product was analyzed using electrophoresis on a 2% agarose gel stained with 2.5% GelRed^{TM} (Biotium, Hayward, USA). The AA genotype was identified when a 264-bp fragment was observed in the electrophoresis of the A allele tube, and the TT genotype was identified with the presence of a 264-bp fragment for the T allele tube. For the AT genotype, one 264-bp fragment was observed in each of the two reaction tubes (A and T).

2. 3 Determination of ancestry

Individual ancestry estimates were based on a panel of 48 insertion-deletion (InDel) ancestry informative markers (AIMs) as described in Santos et al. (2010). The ancestry data for the samples from Goianésia do Pará were previously presented in a larger subset of samples in Cassiano et al. (2015) [46]. The AIMs were genotyped in three multiplex reactions with 16 markers in each reaction, and electrophoresis was performed on a capillary sequencer (ABI®3130 Genetic Analyzer, Applied Biosystems) under the conditions described by De Seixas et al. (2016) [47]. A standard ladder (ABIGS LIZ-500, Applied Biosystems) was used in each sample as a reference for the identification of InDel markers. All of the investigated AIMs significantly differed in frequency in populations of different geographical origins. The individual proportions of European, African and Native American ancestry were estimated in the program Structure v2.3.4 using the Admixture Model with a 100,000 burn length and 100,000 interations after burning; the allele frequencies were independently modeled [48]. For the ancestry estimates, the data obtained in the investigated sample were plotted against the parental population data that formed the Brazilian population, which included Amerindian (246), Western European (290) and Sub-Saharan African (201) individuals. The analysis showed that the main contribution was European (44.2%), but there was also a significant African (31.8%) and Amerindian (24.0%) contribution.

2.4 Statistical analysis

All statistical analyses were performed using R software. The allele, genotype and haplotype frequencies and deviations from the Hardy-Weinberg equilibrium were estimated using the SNPassoc package [49]. Differences in the ancestry proportions between genotypes were determined using fitted logistic regression models for age, gender and infection status. A similar analysis was performed to evaluate differences in ancestry proportions among the different haplotypes using the haplo.glm function [50]. Binary logistic regression was used to graphically explore the associations between the polymorphisms and ancestry proportions using the multinom package [51]. Differences in the genotype and haplotype frequencies between the infected and non-infected individuals were tested using the SNPassoc package with adjustment for the covariates age, gender and ancestry. In all multivariate analyses, the SNPs were included following different genetic models (co-dominant, recessive, dominant and additive). P-values <0.05 were considered significant.

3. Results

3.1 Epidemiological characteristics of the study participants

The demographic data of the subjects included in the study are listed in Table 1. Of the 141 participants, 90 (63.8%) had mild malaria, and 51 (36.2%) individuals were not infected at the time of collection. The proportion of men was higher in the group with malaria (74.4%) than in group of non-infected individuals (56.9%) ($p = 0.03$). Additionally, the proportion of individuals that reported previous episodes of clinical malaria was higher in the group of malaria-infected individuals (91.1% vs 68.6%, $p < 0.01$). Age, number of previous malaria episodes and proportion of genetic ancestry (European, African and Native American) were similar between the two groups. There were no differences in the proportions of African, European and Native American ancestry between the men and the women ($p = 0.99$, 0.65 and 0.48, respectively, Mann–Whitney U-test).

3.2 Genotype and haplotype distributions

The genotype and allele distributions of the studied SNPs are shown in Table 3. The *IFNG*+874A>T SNP was successfully genotyped in 92.2% of the samples; the other SNP in the *IFNG* gene (-183G>A) was removed from the analysis because it was monomorphic. When the allele and genotype frequencies of the remaining six SNPs were compared between malaria-infected and non-infected individuals, no significant association was observed. All SNPs were at Hardy-Weinberg equilibrium in both groups (all p-values > 0.05) (Table 2). We conducted the tests following the additive, dominant, recessive and heterozygous models, and the lowest p-values are shown in Supplementary Table 1. Although the highest *AA* genotype frequency was observed for the *IFNG*+874A>T SNP in the group of malaria-infected individuals, this difference did not reach the significance level (OR = 1.87, 95% CI: 0.91-3.82, p = 0.08).

Haplotype analyses were performed for the three SNPs in the *TNF* gene and for the two SNPs in the *IL10* gene. Four haplotypes in the *TNF* gene were responsible for more than 98% of all potential combinations. The *TNF*-1031T>C SNP was in moderate linkage disequilibrium with the *TNF*-308G>A and -238 G>A SNPs (D' = 0.70 and 0.67, respectively), whereas the *TNF*-308G>A and -238 G>A SNPs exhibited a D' of 0.85. For the *IL10* gene, strong linkage disequilibrium occurred between the -819C>T and -592C>A SNPs (D' and three haplotypes were observed. The comparison of the haplotype frequencies between the malaria-infected and non-infected individuals is shown in Table 3; no significant differences were observed (all p-values > 0.06).

3.3 Association between polymorphisms and genetic ancestry

The individual proportions of the African, European and Native American genetic ancestries were analyzed as continuous variables. In the present study, no differences were observed in the mean proportion of any ancestry among the different genotypes and haplotypes analyzed (Table 4 and Supplementary Table 2). Figure 1 shows the graphical representation of the binary logistic regression model used to evaluate the frequency of individuals carrying the mutant allele of all analyzed SNPs in relation to the individual

genetic ancestry proportions. The frequency of individuals carrying the *TNF-308A* allele progressively decreased with the increasing proportion of European ancestry (p = 0.03). However, when the Bonferroni correction for multiple tests was used, this association was no longer significant (p = 0.18). No other association was observed.

Table 1. Characteristics of the study population.

Characteristic	Mild vivax malaria (n = 90)	Non-infected (n = 51)	*p*-value
Gender, male[a]	74.4	56.9	0.03
Age (years)[b]	32.5 (23.75-43.5)	37.0 (26.0-45.0)	0.62
Genetic ancestry[c]			
European	0.442 ± 0.130	0.449 ± 0.130	0.76
African	0.318 ± 0.120	0.295 ± 0.112	0.26
Native American	0.240 ± 0.094	0.256 ± 0.111	0.35
Previous malaria episodes[b]	5.0 (2.0-7.0)	2.0 (0-6.0)	0.06
Previous history of malaria[a]	91.1	68.6	< 0.01

[a]Percentage [b]Median (IQR) [c]Mean ± SD

Table 2. Distribution of the genotypes between vivax malaria-infected and non-infected individuals

			Malaria			Non-infected		
Gene	SNP	Genotype	n (%)	MAF	HWE	n (%)	MAF	HWE
IFNG	*+874A>T*	*AA*	39 (48.7)	0.30	0.91	17 (34.0)	0.37	0.08
		AT	34 (42.5)			29 (58.0)		
		TT	7 (8.8)			4 (8.0)		
TNF	*-1031T>C*	*TT*	51 (56.7)	0.28	0.11	24 (47.1)	0.28	0.14
		TC	37 (41.1)			25 (49.0)		
		CC	2 (2.2)			2 (3.9)		
TNF	*-308G>A*	*GG*	69 (76.7)	0.12	0.21	35 (68.7)	0.18	0.69
		GA	21 (23.3)			14 (27.4)		
		AA	0			2 (3.9)		
TNF	*-238G>A*	*GG*	80 (88.9)	0.06	0.22	46 (90.2)	0.05	0.71
		GA	9 (10.0)			5 (9.8)		
		AA	1 (1.1)			0		
IL10	*-819C>T*	*CC*	39 (43.3)	0.34	0.87	19 (37.2)	0.35	0.15
		CT	41 (45.6)			28 (54.9)		
		TT	10 (11.1)			4 (7.8)		
IL10	*-592C>A*	*CC*	41 (45.6)	0.29	0.05	20 (39.2)	0.34	0.21
		CA	45 (50.0)			27 (52.9)		
		AA	4 (4.4)			4 (7.8)		

Abbreviations: MAF, Minor allele frequency; HWE, Hardy-Weinberg equilibrium

Table 3. Haplotype frequencies in the *TNF* and *IL10* genes in vivax malaria-infected and non-infected individuals.

Haplotype	Malaria	Non-infected	OR (95% CI)	*p*-value
TNF$_{-1031/\ -308/\ -238}$				
T/G/G	0.632	0.555	Reference	0.11
C/G/G	0.195	0.220	0.63 (0.30-1.31)	0.45
T/A/G	0.113	0.161	0.48 (0.20-1.13)	0.17
C/G/A	0.037	0.049	0.62 (0.15-2.35)	0.75
IL10$_{-819/-592}$				
C/C	0.642	0.647	Reference	0.94
T/A	0.291	0.343	0.80 (0.44-1.44)	0.33
T/C	0.054	0.009	7.19 (0.89-57.7)	0.06

Odds ratios (OR), 95% confidence interval (CI)

Table 4. Haplotype frequency and its association with the proportions of African, European and Native American ancestry.

		African			European			Native American		
Haplotype	Frequency	Proportion	Difference (95% CI)	*p*-value	Proportion	Difference (95% CI)	*p*-value	Proportion	Difference (95% CI)	*p*-value
TNF$_{-1031/\ -308/\ -238}$										
T/G/G	0.615	0.31	Reference		0.44	Reference		0.25	Reference	
C/G/G	0.191		-0.01 (-0.05 – 0.03)	0.61		-0.01 (-0.06 – 0.03)	0.57		0.01 (-0.02 – 0.05)	0.49
T/A/G	0.121		-0.05 (-0.10 – 0.00)	0.05		0.06 (0.00 – 0.11)	0.05		-0.02 (-0.06 – 0.02)	0.37
C/G/A	0.044		-0.01 (-0.08 – 0.06)	0.78		-0.02 (-0.11 – 0.06)	0.55		0.06 (-0.03 – 0.15)	0.17
IL10$_{-819/-592}$										
C/C	0.650	0.30	Reference		0.45	Reference		0.25	Reference	
T/A	0.303		-0.01 (-0.04 – 0.03)	0.67		0.00 (-0.04 – 0.04)	0.94		0.01 (-0.02 – 0.04)	0.55
T/C	0.040		0.01 (-0.07 – 0.08)	0.86		-0.02 (-0.10 – 0.06)	0.60		0.02 (-0.05 – 0.08)	0.63

The effects of each haplotype were relative to the most frequent haplotype used as a reference. Δ% indicates relative change in the ancestry proportions compared to the reference haplotypes with 95% confidence intervals.

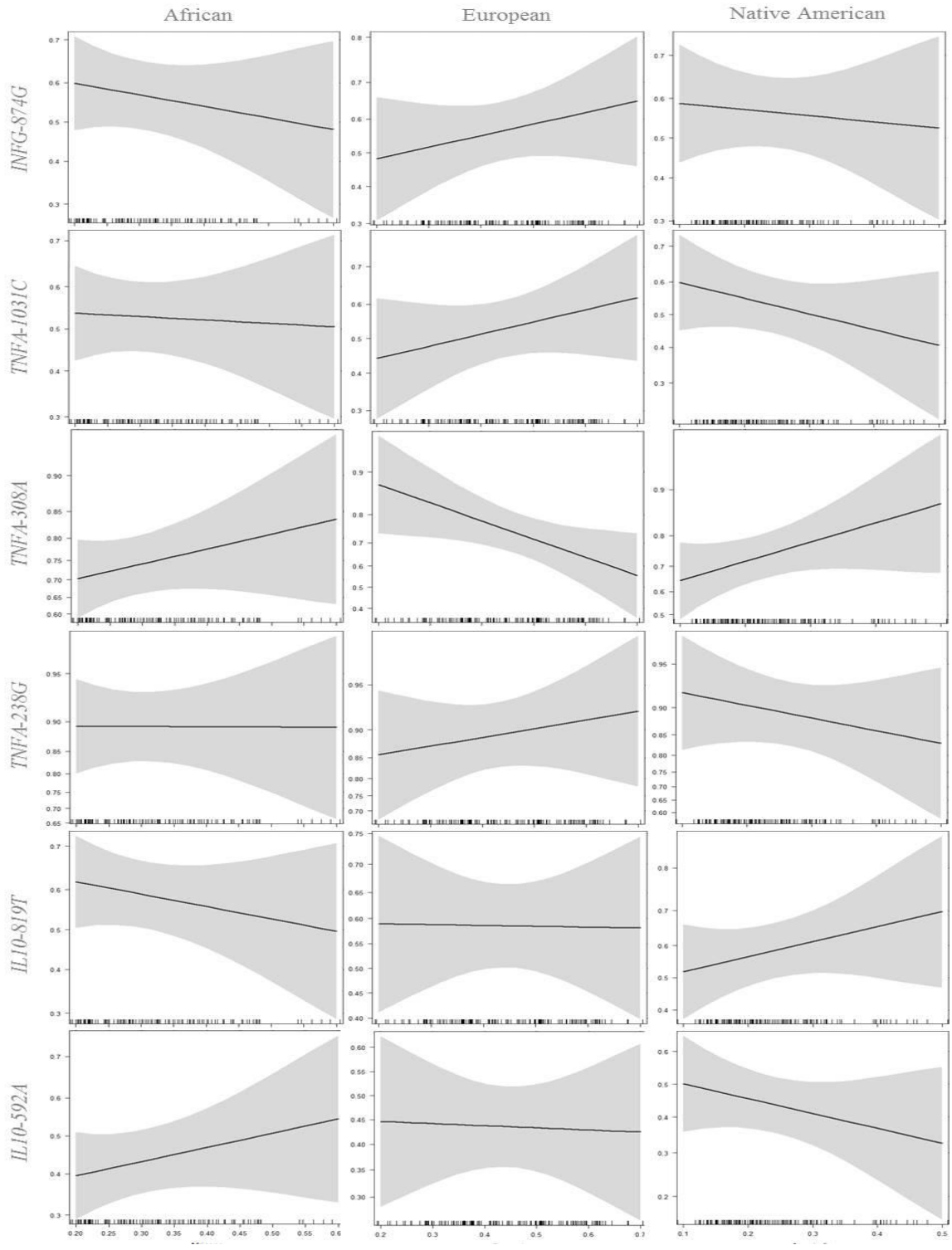

Figure 1. Binary logistic regression model used to evaluate the frequency of individuals carrying the mutant allele of all analyzed SNPs relative to the individual proportions of

genetic ancestry. The shading around the lines represents the 95% confidence interval. The graph was constructed using the ggplot2 package in the R program.

4. Discussion

Previous studies reported different allele frequencies in cytokine genes among different ethnicities. Due to these studies and the participation of these proteins in numerous processes related to the pathogenesis of various diseases, we evaluated the frequencies of polymorphisms in the *TNFA*, *IFNG* and *IL10* genes in a highly admixed Brazilian population and related their distributions to the proportions of genetic ancestry using AIMs. We selected a population from northern Brazil where there was a higher contribution of Native American ancestry due to the lack of data in studies of this nature involving indigenous populations [52]. Because these cytokines play a key role in the modulation of the immune response in malaria, we evaluated whether these polymorphisms were related to protection against vivax malaria. However, this study did not provide evidence of such associations.

The *-308G>A* SNP (rs1800629) is located in the promoter region of the gene, and the presence of the *A* allele forms a binding site for the AP1 transcription factor that has been associated with increases in TNF-α production [18]. The frequency distribution of the *A* allele observed in our study (13.83%) was similar to that observed in previous studies in the Brazilian population (12-16%) [53, 54, 55]. According to data from the 1000Genomes project, the frequency of the *A* allele is similar between Europeans (13%) and Africans (12%). This finding was in agreement with our results because no differences were observed in the frequencies of this allele according to the proportions of genetic ancestry. Contradictory results were observed for malaria, with the *TNF-308A* allele associated with higher susceptibility/severity [56,57,58], without alterations [36] or with resistance to *P. falciparum* malaria [59]. Regarding vivax malaria, which was the focus of the present study, our results were in agreement with other studies, including those in the Brazilian Amazon that did not observe any associations between the *TNF-308G>A* SNP and susceptibility or clinical manifestations due to *P. vivax* infection [10, 19, 60].

The -238 G/A SNP *(rs361525)* does not have a clearly established function but seems to affect the circulating cytokine levels because it is located on a repressor site in the TNF-α gene [16]. The 5.38% frequency of the A allele (-238) in our results was similar to the data for Europeans and Africans, which ranged from 4 to 6% [61]. The frequency of the presence of the A allele at the -238 and -376 positions is low worldwide. In the Brazilian Amazon, previous indices ranged from 5-7% [19, 62], and no associations were described with vivax malaria in Pará [19]. In contrast, the G/A genotype was associated with psoriasis in southeastern Brazil [63], and the A allele was associated with a decrease in falciparum malaria parasitemia in Burkina Faso [30], cerebral malaria in Kenya [64] and malarial anemia [56]. This SNP was associated with increased susceptibility to vivax malaria in the Amazon region only when evaluated in the TATGG haplotype (−1031/-863/-857/-308/-238) [19].

The 24.82% frequency of the C allele at the -1031 position (*rs1799964*) of the *TNFA* gene is similar to data from the 1000Genomes project (15% and 21% for Africans and Europeans, respectively) and Brazilian studies on leprosy and vivax malaria [62]. In malaria, this SNP was associated with cytokine levels and clinical symptoms but not with susceptibility in India [27]. The C allele is associated with a two-fold higher chance of cerebral malaria caused by *P. falciparum* [65] in Thailand. In Africa, the CC genotype is associated with repeated malaria episodes [44,59] and the T allele is associated with high parasitemia [30]. In Brazil, the CC genotype is associated with protection against leprosy but not malaria [62].

One hypothesis for the lack of association of the evaluated SNPs is that malaria can occur due to possible linkage disequilibrium of the SNPs in *TNFA* with the human leukocyte antigens (HLAs), which can cause non-functional mutations [59, 60]. The A allele (-308) is described as having a strong linkage disequilibrium with HLA-Bw53 and DRB1*1302-DQB1*0501, whereas the A allele at the -238 position of the *TNFA* gene appears to be linked to HLA-B53 but with different immune characteristics [56]. The haplotype frequencies in cytokine genes can vary extensively among different ethnic groups most likely due to selective pressure on the human genome and thus affect the

susceptibility and clinical outcomes of diseases such as malaria [33]. This effect might have affected our results due to the admixture observed in the Brazilian population.

. The gene sequence of this cytokine is highly conserved, with few polymorphisms. The SNP at the -183 G/T position is related to increased transcription activity [26], whereas +874 (A>T) is located in a region where the number of replicates can modulate the expression of messenger RNA and the production of cytokines [21,66]. The T allele is associated with a high number of replicate copies and activates the transcription site for the NF-κB pathway, which correlates with high cytokine expression [67, 68]. The A/A, T/A and T/T genotypes are associated with low, intermediate and high production of *IFN-γ*, respectively [21,69].

The highest frequency of the A allele (*IFNG*+874) is described in individuals with European ancestry and is 46% (http://hapmap.ncbi.nlm.nih.gov). Indeed, the evaluated population in the present study had a European contribution of almost 50% [43], and the frequency of this allele was detected in 67.3% of the evaluated sample. However, no association was detected with any ancestry or with malaria. Studies conducted in the United States with African-American and Caucasian populations found higher frequencies of 66% and 37% [70] -and 48% and 25% [71], respectively. Our data showing the higher frequency of the mutant A allele are in agreement with studies in the Brazilian Amazon that found frequencies of 70.13% [21] and 73% [17], but all lacked an association with malaria caused by *P. vivax* or *P. falciparum*. Few studies have described an association between this SNP with malaria; however, its association with dermatitis was observed in India [72] and with an increase in susceptibility to malaria in Brazil [21]. Importantly, higher levels of this cytokine allow a better immune response against obligate intracellular pathogens; thus, low levels of the A allele are associated with susceptibility to the disease.

The *IL10* gene has more than 27 polymorphic sites associated with SNPs that result in the differential production and expression of the cytokine [17, 33, 73], auto-immune and inflammatory diseases [74], bacterial and viral infections [75] and human malaria [21]. Particularly, -819C and -1082G increase the protein production in peripheral blood

lymphocytes *in vitro* [76]. The (-1082, -819 and -592) GCC, ACC and ATA haplotypes are associated with high, intermediate and low IL-10 production, respectively [17, 33].

The allele distributions for T (-819) and A (-592) in our results were 35.4% and 31.2%, respectively; these distributions were higher in Europeans than in Africans but lacked significant associations. These data disagree with those from the 1000Genomes project [61], which reports a higher frequency of the mutant alleles in Africans. Lokossu et al. 2013 [77] reported higher frequencies (41.53% and 41.31% for the T and A alleles, respectively) for falciparum malaria in Benin. The allele and genotype distributions of SNPs in *IL10* are described as variables according to ethnic group [21, 33] and the A (-592), T (-819) and A (-1082) alleles are more frequent among African-Americans [77,78]. Moraes et al. 2003 [74] also found no associations of genotypes, alleles and haplotypes with five *IL10* SNPs (–3575, –2849, 2763, –1082, –819) in a study with Brazilian and Dutch populations. However, studies with indigenous populations are scarce. In Brazil, a study with the Terena of Mato Grosso do Sul state showed that the mutation rate was significant for the *IL10* -819 and -1082 SNPs [79]; in contrast, we obtained the lowest rates for this ancestry and no association was observed.

The CC genotypes for the two SNPS were associated with a decrease in IL-10 levels and low parasitemia in northern Brazil [17], which agreed with our data indicating no significant association with susceptibility to malaria. Two studies in Pará state, Brazil, also described no haplotype associations of the *IL10* gene with malaria [19, 21] and falciparum malaria in Africa [33]. In Piracicaba, southeastern Brazil, these SNPs were associated with chronic periodontitis in Caucasians [80]. Future analyses of parasitemia and cytokine indices may identify associations between the SNPs in the evaluated sample. One hypothesis for the lack of association is that the patients involved in the present study did not have malarial complications caused by *P. vivax*. Additionally, the transmission profile of the malaria of the area investigated could have had an effect, and the epidemiology was different from that observed in Africa. Another explanation may be the low frequency of some genotypes in the present study. Thus, the sample size may have been too small to find any possible association. This finding warrants further investigation.

Conclusion

The evaluation of ancestry informative markers (AIMs) allows estimations of admixtures at the individual level and avoids possible confounding factors due to ethnicity, such as in the tri-hybrid population sample evaluated in this study. Although most polymorphisms in the *TNFA, IFNG* and *IL10* genes investigated in this study did not significantly differ according to ancestry and were not associated with risk or protection against vivax malaria, the A allele of TNF-308 progressively decreased with the increasing proportion of European ancestry. In Brazil, this is the first study to evaluate the distribution of these genes according to ancestry. The results support the application of ancestry informative markers in future studies.

Conflict of Interests

None of the authors declare a conflict of interests.

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RESEARCH

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Cytokine gene polymorphisms are not associated with anti-PvDBP, anti-PvAMA-1 or anti-PvMSP-1_{19} IgG antibody levels in a malaria-endemic area of the Brazilian Amazon

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Abstract

Background: The immune response against *Plasmodium vivax* immunogenic epitopes is regulated by pro- and anti-inflammatory cytokines that determine antibody levels and class switching. Cytokine gene polymorphisms may be responsible for changes in the humoral immune response against malaria. The aim of this study was to evaluate whether polymorphisms in the *TNFA*, *IFNG* and *IL10* genes would alter the levels of anti-PvAMA1, PvDBP and -PvMSP-1_{19} IgG antibodies in patients with vivax malaria.

Methods: Samples from 90 vivax malaria-infected and 51 uninfected subjects from an endemic area of the Brazilian Amazon were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) to identify polymorphisms of the genes *TNFA* (−1031T > C, −308G > A, −238G > A), *IFNG* (+874T > A) and *IL10* (−819C > T, −592C > A). The levels of total IgG against PvAMA1, PvDBP and PvMSP-1_{19} were determined using an enzyme-linked immunosorbent assay (ELISA). Associations between the polymorphisms and the antibody response were assessed by means of logistic regression models.

Results: No significant differences were found in the levels of IgG antibodies against the PvAMA-1, PvDBP or PvMSP-1_{19} proteins in relation to the studied polymorphisms.

Conclusions: Although no associations were found among the evaluated genotypes and alleles and anti-merozoite IgG class *P. vivax* antibody levels, this study helps elucidate the immunogenic profile involved in the humoral immune response in malaria.

Keywords: IgG antibody, *Plasmodium vivax*, TNF, IFNG, IL10

Background

Early diagnosis, prompt and effective treatment, the use of mosquito nets impregnated with long-acting insecticides and residual intradomiciliary spraying are the main malaria control measures [1] and have resulted in a reduction in the transmission and number of cases of malaria in Brazil. However, this disease remains one of the most prevalent infections in tropical countries, with 214 million clinical cases/year and approximately 438,000 deaths [2]. In Brazil, *Plasmodium vivax* is the aetiologic agent in 85 % of cases, and 99.9 % of cases occur in the Brazilian Amazon region [2].

The different clinical manifestation patterns of malaria may be related to host and agent genetic factors, age and ethnicity as well as the involvement of these factors in the immune response and parasitaemia and antibody levels [3–5]. IgG antibodies play a protective role against parasite invasion in the erythrocytic cycle of *Plasmodium* [3, 6]. For *Plasmodium knowlesi,* anti-PvAMA-1 monoclonal

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antibodies have been shown to be capable of inhibiting merozoite invasion in vitro [7]. PvDBP antibodies inhibit interactions with the Duffy antigen receptor for chemokines (DARC) in erythrocytes [8], and antibodies produced against PvMSP-1_{19} have been shown to prevent merozoite invasion in vitro [9]. The synthesis of immunoglobulins is complex and depends on the process of antigen presentation by B lymphocytes (BL) to TCD4 lymphocytes and the involvement of co-stimulatory molecules and their receptors [10].

The production, levels and switching of antibody classes is regulated by pro- and anti-inflammatory cytokines [3, 6, 11, 12]. IFN-γ can negatively modulate the humoral immune response, thus interfering with circulating antibody levels [13] and increasing IgG2 production. TNF appears to be important in the development of the humoral response as an autocrine growth factor for B cells [6]. Among the anti-inflammatory cytokines, IL-10 may participate in negative immunomodulation of the Th1-type response [14, 15] in addition to inducing immunoglobulin synthesis [6].

Many genetic variants are responsible for minor changes in the immune response in malaria [11, 12]. Previous studies in Brazil with vivax malaria have evaluated factors associated with genetic variability in the humoral immune response. HLA-DR16 is associated with the IgG antibody response to the *P. vivax* VK247 variant circumsporozoite protein [16]. High levels of MSP-1 antibodies are also associated with HLA-DR3 [17]. In Goianésia do Pará, a municipality located in the Brazilian Amazon, two studies evaluated the effects of polymorphisms in genes involved in the humoral immune response. Cassiano et al. [10] found that the frequency of specific IgG responders against PvAMA-1, PvDBP and PvMSP-1_{19} was associated with polymorphisms in the *BLYS* (−871C > T), *CD40* (−1C > T) and *CD86* (+1057G > A) genes. In contrast, genotypes and haplotypes of the *IL4* gene were not associated with the production of PvAMA-1 antibodies [18]. The aim of this work was to continue the search for the genetic basis of these traits and to evaluate whether polymorphisms in the *TNFA, IFNG* and *IL10* genes alter the levels of anti-PvAMA1, -PvDBP and -PvMSP-1_{19} IgG antibodies.

Methods

Study area and subjects

Ninety samples from vivax malaria-infected subjects and 51 samples from uninfected subjects were collected in Goianésia do Pará (03°50′33″S; 49°05′49″ W). The subjects were in a sub-group of individuals previously analysed by Cassiano et al. [10]. The study has evaluated the effect of genetic ancestry on the distribution of polymorphisms in the *TNFA*, *IFNG* and *IL10* genes (unpublished data). No differences were observed in the mean proportion of any ancestry among the different genotypes and haplotypes analysed.

In 2011 and 2012, the numbers of malaria cases were 2856 and 1136, respectively, with 79 % of cases caused by *P. vivax*. Samples were collected at the municipal health center between February 2011 and August 2012. Data were collected by passive detection in Basic Health Units after thick blood film phenotypic diagnosis, but prior to treatment. All patients if malaria were given standard treatment of 1500 mg of chloroquine in 3 days (600, 450 and 450 mg) plus 30 mg of primaquine on the day the diagnosis and on the following 6 days. Individuals infected with *P. vivax* presented for medical care because of clinical symptoms of malaria and they were recruited after diagnosis. The uninfected individuals who sought medical care offered during the study were invited to participate in the study. These individuals experienced the same conditions of exposure to the aetiological agent. All participants or guardians signed the consent form, and the project was approved by the Goianésia do Pará health authorities and by the Research Ethics Committee (CAAE 01774812.2.0000.5415) of the College of Medicine of São José do Rio Preto (Faculdade de Medicina de São José do Rio Preto).

Malaria was diagnosed using thick blood smears stained with Giemsa and subsequently confirmed by nested-PCR with modifications [19]. No uninfected individual was positive in nested-PCR, while five individuals positive for *P. vivax* (thick blood smear) had mixed infection with *Plasmodium falciparum* (by nested-PCR) and were excluded from the study. Parasitaemia was defined as the number of parasites per microlitre of blood after examination of 100 microscopic fields.

Peripheral blood was stored at −20 °C. The examination of polymorphisms in the genes *TNFA, IFNG* and *IL10* was performed via PCR amplification followed by restriction fragment length polymorphism (RFLP) analysis or the amplification of specific alleles (Table 1).

The amplified products were subjected to electrophoresis (100 V/50 min) in 1.5 to 2.5 % agarose gels stained with Gel Red (Biotium, CA, USA) or in ethidium bromide-stained 12.5 % polyacrylamide gels (10 mg/ml) and were visualized using a transilluminator (Biotecnologia-Locus).

Assessment of the serological response against *Plasmodium vivax*

The levels of IgG class anti-MSP-1_{19}, anti-PvAMA-1 and anti-PvDBP antibodies in a larger cohort were previously reported by Cassiano et al. [10]. The analyses were performed using ELISA following previously described protocols [24–26]. A recombinant protein (His6-MSP-1_{19})

Table 1 Polymorphisms, methods, restriction enzymes, primers, and band patterns used for investigation of SNPs in genes *TNFA, IFNG, IL10*

Polymorphisms in gene	SNP	Method enzimas annealing	Primers	Genotype	Reference
IFN γ −183 G > T	*rs2069709*	*RFLP* (53º) *Eco47I*	FW: 5′-AAT GAT CAA TGT GCT TTG TG-3′ R: 5′-TAA GAT GAG ATG GTG ACAG-3′	TT: 271 pb GT: 271 pb, 164 pb, 107pb GG: 164pb, 107 pb	Suxia Qi et al. [20]
IFN γ +874 A > T	*rs2430561*	ASO-PCR (53º)	CP: -5′-TCA ACA AAG CTG ATA CTC CA-3′ T : 5′-TTC TTA CAA CAC AAA ATC AAA TCT-3′ A: 5′-TTC TTA CAA CAC AAA ATC AAA TCA-3′	AA: 262 pb (reação A) TT: 262 pb (reação T) AT: 262 pb (reação A, T)	Medina et al. [21]
TNF α −238 G > A	*rs 361525*	RFLP (60º) *MspI*	FW: 5′-ATC TGG AGG AAG CGG TAG TG-3′ R: 5-AGA AGA CCC CCC TCG GAA CC′3′	AA: 156 pb GG: 133 pb GA: 153 pb, 133 pb	Hedayati et al. [22]
TNFα −308 G > A	*rs 1800629*	RFLP (59º) *NcoI*	FW: 5′-GAG GCA ATA GGT TTT GAG GGC CAT-3′ R: 5′-GGG ACA CAC AAG CAT CAAG 3′	AA: 147 pb GG: 126 pb, 121 pb GA: 147 pb, 126 pb, 21 pb	Asghar et al. [23]
TNFα −1031 T > C	*rs1799964*	RFLP (57º) *BbsI*	FW: 5′-TAT GTG ATG GAC TCA CCA GGT-3′ R: 5′-CCT CTA CAT GGC CCT GTC TT 3′	TT: 251 pb, 13 pb TC: 251 pb, 180 pb, 71 pb e 13 pb CC: 180 pb, 71 pb e 13 pb	Asghar et al. [23]
IL-10 −592 C > A	*rs1800872*	RFLP (54º) *RsaI*	FW: 5′-GGG TGA GGA AAC CAA ATT CTC-3′ R:5′-GAG GGG GTG GGC TAA ATA TC 3′	AA: 240 pb, 77 pb, 36 pb e 08 pb CC: 317 pb,36 pb e 08 pb CA: 317 pb, 240 pb, 77 pb, 36 pb e 08 pb	
IL-10 −819 C > T	*rs1800871*	RFLP (54º) *RseI*	FW: 5′-GGG TGA GGA AAC CAA ATT CTC-3′ R: 5′-GAG GGG GTG GGC TAA ATA TC 3′	TT: 270 pb e 91 pb CC: 217 pb, 91 pb e 53 pb TC: 270 pb, 217 pb, 91 pb e 53 pb	

comprising amino acids 1616–1704 of MSP-1_{19} [24] and domain II of the DBP protein [25] of *P. vivax* (SAll strain) was expressed in *Escherichia coli*. A protein ectodomain (amino acids 43–487) expressed in *Pichia pastoris* was used for PvAMA-1 [26]. The reactivity index (RI) was calculated to define samples as reactive or non-reactive and was determined by dividing the sample OD value by the cut-off point. Samples with RI ≥ 1 were considered positive, and those with RI < 1 were considered negative. The cut-off point was established as the mean OD (plus three standard deviations) of the 40 plasma samples collected from subjects with no history of malaria who were living in São José do Rio Preto, which is located in the interior of the state of São Paulo (a non-malaria-endemic area). The control samples were only used for the determination of serological response.

Statistical analysis

Statistical analysis was performed using R software v 2.11.1. The genotypic and allelic frequencies of each variant were calculated using the genetics package [27]. This package was used to evaluate Hardy–Weinberg equilibrium deviations using the Chi square test and the linkage disequilibrium between locus pairs was calculated using the D′ parameter. Haplotypes frequencies were estimated using the maximum likelihood method with the EM (expectation–maximization) algorithm, which is part of the haplo stats package [28]. Differences between the proportions of responders and non-responders were evaluated using the Chi square test. A non-parametric Kruskal–Wallis test was used to identify differences in antibody levels and genotypes. *P* values less than 0.05 were considered significant.

Results

The genotypic frequencies of the six examined single nucleotide polymorphisms (SNPs) are summarized in Table 2. The allelic frequencies of the evaluated SNPs were in Hardy–Weinberg equilibrium. For the *IFNG* gene, allele A was the most frequent, with 67.3 % at position +874T > A. For positions −592C > A and −819C > T of the *IL10* gene, the C allele was most common, with frequencies of 68.8 and 65.6 %, respectively. Finally, for the *TNFA* gene at positions −238G > A,

Table 2 Levels of IgG antibodies against *P. vivax* blood stage proteins according to the studied genotypes

Gene	SNP	PvAMA-1 (n = 135)	*P* value	PvDBP (n = 135)	*P* value	PvMSP-1_{19} (n = 128)	*P* value
INFG	*+874T > A*		0.19		0.40		0.42
	AA	1.36 (0.63–2.78)		1.58 (0.78–7.29)		2.92 (1.00–7.63)	
	AT	1.14 (0.51–2.18)		1.41 (0.77–4.73)		2.05 (0.49–6.64)	
	TT	2.50 (1.18–3.00)		5.17 (0.86–18.5)		4.66 (1.50–8.11)	
TNFA	*−1031T > C*		0.74		0.42		0.71
	TT	1.41 (0.60–2.97)		1.34 (0.79–5.38)		3.74 (0.96–7.57)	
	TC	1.49 (0.58–2.50)		1.66 (0.79–6.87)		2.60 (0.51–6.93)	
	CC	1.45 (1.01–1.52)		8.57 (0.73–14.89)		2.12 (1.32–2.59)	
TNFA	*−308G > A*		0.41		0.58		0.50
	GG	1.67 (0.65–2.80)		1.66 (0.79–6.30)		2.92 (0.89–7.58)	
	GA	1.14 (0.58–2.23)		1.48 (0.73–3.42)		2.61 (1.18–6.54)	
	AA	0.62 (0.51–0.62)		1.39 (0.96–1.39)		1.30 (0.28–1.30)	
TNFA	*−238G > A*		0.76		0.97		0.61
	GG	1.45 (0.64–2.66)		1.59 (0.81–5.59)		2.61 (0.94–7.47)	
	GA	1.34 (0.53–2.91)		1.48 (0.76–7.16)		5.15 (1.69–8.16)	
	AA	2.32 (2.32–2.32)		1.28 (1.28–1.28)		3.04 (3.04–3.04)	
IL10	*−819C > T*		0.79		0.57		0.99
	CC	1.35 (0.63-2.53)		2.01 (0.79–5.91)		3.50 (0.55–7.48)	
	CT	1.45 (0.60-2.77)		1.29 (0.77–5.06)		2.61 (0.87–7.60)	
	TT	2.22 (0.83–2.84)		2.18 (1.35–6.31)		1.98 (1.22–7.13)	
IL10	*−592C > A*		0.86		0.86		0.77
	CC	1.41 (0.62–2.50)		1.59 (0.76–5.91)		4.92 (1.10–7.63)	
	CA	1.52 (0.62–2.78)		1.46 (0.81–5.46)		2.59 (0.94–7.26)	
	AA	1.22 (0.52–2.85)		1.84 (1.21–4.32)		2.50 (1.07–5.97)	

Values are presented as medians (IQ)

−308G > A and −1031T > C, the most frequent alleles were G (94.32 %), G (86.17 %) and T (75.18 %).

Antibodies levels (PvAMA-1, PvDBP or PvMSP-1_{19}) were significantly higher in individuals with malaria than in uninfected (Additional file 1). No significant differences were found in the levels of IgG antibodies against the PvAMA-1, PvDBP or PvMSP-1_{19} proteins in relation to the studied polymorphisms (Table 2, $P > 0.05$, Kruskal–Wallis test). It was possible to genotype some samples of patients with malaria to the IFNG (+874A > T) gene rs2430561. This result did not change in the regression analyses after adjusting for covariates affecting antibody levels (current infection status, previous history of malaria and gender). The logistic regression analyses also revealed no significant differences between haplotypes in relation to antibody levels (Table 3).

Discussion

Cytokines such as TNF, IFN-γ and IL-10 participate in cellular and humoral immune modulation in malaria and affect pathogenesis, parasitaemia control and pathophysiology, which are dependent on the cellular and circulating levels of these cytokines [3, 21, 29, 30]. It is hypothesized that polymorphisms in the genes encoding these cytokines could be found in genetic markers and would affect the levels of IgG anti-merozoite antibodies in individuals with vivax malaria. Limited data are available in the literature, and studies examining associations between antibodies and SNPs in cytokine genes in general have been conducted only for *P. falciparum*. The aim of the present study was to evaluate the importance of polymorphisms in the genes *TNFA*, *IFNG* and *IL10* in the antibody response of *P. vivax* vaccine candidate proteins. However, analysis identified no significant association.

IFN-γ is a key pro-inflammatory cytokine for the induction of essential immune effector mechanisms in initial infection control in both the hepatic and erythrocytic phases of malaria [31, 32]. The production of this cytokine is related to low parasitaemia in the acute phase; however, a balance with anti-inflammatory cytokines, such as IL-10 and TGF-beta, is necessary to reduce severe forms of malaria [32]. The SNP at position +874 T > A affects an NFkB pathway that determines the production of inflammatory cytokines [33, 34]. An association has

Table 3 Frequency of haplotypes and their association with levels of IgG antibodies against the PvAMA-1, PvDBP and PvMSP-1$_{19}$ proteins

Haplotypes	Frequency	PvAMA-1			PvDBP			PvMSP-1$_{19}$		
		RI estimation	Difference (95 % CI)	*P* value	RI estimation	Difference (95 % CI)	*P* value	RI estimation	Difference (95 % CI)	*P* value
TNF$_{-1031/-308/-238}$										
T/G/G	0.606	1.76	Reference		4.01	Reference		4.35	Reference	
C/G/G	0.201		−0.13 (−0.49 to 0.24)	0.50		1.86 (−0.15 to 3.86)	0.07		−0.62 (−1.75 to 0.52)	0.29
T/A/G	0.127		−0.31 (−0.74 to 0.12)	0.15		−0.89 (−3.20 to 1.41)	0.45		−0.58 (−1.88 to 0.73)	0.39
C/G/A	0.041		−0.07 (−0.78 to 0.63)	0.84		−1.51 −5.17 to 2.15)	0.42		0.19 (−1.99 to 2.36)	0.86
IL10$_{-819/-592}$										
C/C	0.644	1.54	Reference		4.77	Reference		4.13	Reference	
T/A	0.311		0.05 (−0.25 to 0.35)	0.74		−0.09 (−1.83 to 1.66)	0.92		−0.48 (−1.42 to 0.46)	0.32
T/C	0.037		0.50 (−0.17 to 1.18)	0.14		−1.50 (−5.43 to 2.43)	0.45		1.34 (−0.75 to 3.44)	0.21

The effects of each haplotype were relative to the most frequent haplotype, which was used as the reference

Δ % indicates the relative change in antibody levels compared to reference haplotypes, with a confidence interval of 95 %

been observed between the T allele of this SNP and high IFN-γ production [34]. The results of individuals carrying the TT (Table 2) genotype had higher levels of anti-PvAMA-1, -PvMSP-1_{19} and -PvDBP IgG antibodies than those with the TA or AA genotypes, although the association was not significant. Thus, the TT genotype may not be related to increased production of IFN-γ, which would negatively modulate the Th2 immune response and antibody production in the analysed samples, given that these patients had higher antibody levels.

TNF participates in the total IgG response, which is mediated by follicular dendritic cells and dependent on soluble TNFR1 signalling [35], and high levels of this cytokine are related to malarial paroxysm [32] and severe malaria [6]. The A allele of *TNFA* (−308, −238) has been associated with elevated levels of antibodies in falciparum malaria [6, 35, 36]. At positions −863 and −857, the A and T alleles, respectively, have been associated with high levels of IgG3 and IgG4 antibodies in malaria [37]. In Tanzania, Carpenter et al. [11] reported a negative association between levels of anti-*P. falciparum* IgG antibodies and the A allele (−308) in malaria patients. In a study of SNPs in the *TNFA* gene in Burkina Faso, the A (−863), T (−857) and G (−1304) alleles in particular were associated with total IgG levels against *P. falciparum*; however, no association was found for positions −1031, −308 or −238 of this gene [6]. This prevalence of subjects individuals with the A allele (*TNF*308G > A) had the lowest levels of anti-PvAMA-1, -PvMSP-1_{19} and -PvDBP IgG antibodies, but the association was not significant.

Interleukin 4, IL-10 and IL-13 are anti-inflammatory cytokines involved in antibody production mechanisms [3]. IL-10 is a Th2-type immunoregulatory cytokine that negatively modulates the effects of pro-inflammatory cytokines produced by Th1 cells [29, 30, 38], participates in the induction of immunoglobulin synthesis and promotes isotype class switching from IgM to IgG1 and from IgG1 to IgG3 [6]. The AA genotype (−1082) has been associated with high levels of anti-MSP-2/31D7 and -AMA-1 IgG antibodies in mothers and newborns with falciparum malaria in Uganda [3]. In patients with falciparum malaria in Tanzania, the A allele (−592, −1082) was associated with low levels of IgE and IgG4 [11]. SNPs (−592 and −819) were associated with high levels of IgE and *P. falciparum* NANP (IgG) antigen antibodies in Sri Lanka [39]. Haplotypes IL10−1082/−819/−592 GCC, ACC and ATA were correlated with high, intermediate and low levels, respectively, of the IL-10 cytokine [29, 40]. It has been hypothesized that high cytokine concentrations are associated with the presence of the C allele at positions −819 and −592 based on the observation that IL-10 participates in the immunological activation of antibody production. However, this proposition was supported for the analyses carried out only for anti-PvMSP-1_{19} IgG for these genotypes. The opposite occurred in the presence of the TT (−819) and AA (−592) genotypes associated with low production of anti-MSP-1_{19}; however, there was no statistical significance in either case. This finding may be due to low serum concentrations of the cytokine resulting from other factors or to the non-influence of these SNPS on IL-10 levels and antibodies in the patients evaluated in this study.

Conclusions

This study revealed no association between genotypes and alleles with IgG class *P. vivax* anti-merozoite antibody levels. Studies of possible associations between SNPs in cytokine genes and the humoral immune response to malaria are still incipient, and the results are contradictory. However, this was the first Brazilian study to examine this set of SNPs (*IFNG-183G > T, +874A > T; TNFA −238G > A, −308G > A and −1031T > C; IL10-592C > A, −819C > T*) in control cases of vivax malaria. Immunogenetic profile studies are needed to better understand the immunomodulation of *P. vivax*; this research will be essential for the development of new malaria vaccines and treatments.

Additional file

Additional file 1. IgG antibody levels to PvAMA-1, PvDBP and PvMSP-1_{19}.

Abbreviations

BL: lymphocytes B; BLYS: B-lymphocyte stimulator; CD40: CD40 gene; CD86: CE86 gene; DARC: duffy antigen receptor for chemokines; ELISA: enzyme-linked immunosorbent assay; HLA-DR3: human leukocyte antigen-DR3; HLA-DR16: human leukocyte antigen-DR16; IgE: immunoglobulin E; IgG: immunoglobulin G; IgG1: immunoglobulin G, subclass 1; IgG2: immunoglobulin G, subclass 2; IgG4: immunoglobulin G, subclass 4; IL4: interleukin 4; IL-10: interleukin 10; *IL10*: interleukin 10 gene; IL-13: interleukin 13; *INFG*: interferon gamma gene; INFγ: cytokine interferon gamma; LTCD4: lymphocyte CD4 T; MSP-2/31D7: merozoite surface protein–2/31D; PvAMA-1: apical membrane antigen-1; PvDBP: duffy binding protein; PvMSP-1_{19}: merozoite surface protein-1; RFLP: restriction fragment length polymorphism; RI: reactivity index; TNF: cytokine tumour necrosis factor; *TNFA*: tumour necrosis factor gene; VK247: variant of *Plasmodium vivax*.

Authors' contributions

AACF, GCC and RLDM contributed to the design and conception of the study and wrote the manuscript. MPC participated in the molecular analyses. MGC and GCC performed the serum analyses. GCC performed the statistical analyses. AACF, GCC and RLDM contributed to the data interpretation and reviewed the manuscript. All the authors read and approved of the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
Biological samples used for this project and preparation of the manuscript are frozen and can be used for further analysis if requested.

Ethics approval and consent to participate
All participants or guardians signed the consent form, and the project was approved by the Goianésia do Pará health authorities and by the Research Ethics Committee (CAAE 01774812.2.0000.5415) of the College of Medicine of São José do Rio Preto (Faculdade de Medicina de São José do Rio Preto).

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CONCLUSÕES

CONCLUSÕES

As seguintes conclusões puderam ser obtidas a partir da amostragem populacional avaliada:

-Os SNPs avaliados estão em Equilíbrio de Hardy-Weinberg.

- As frequências alélicas e genotípicas dos SNPs avaliados são compatíveis com investigações de outras regiões brasileiras e não mostram diferenças entre indivíduos com malária e os não infectados.

- O SNP no gene *INFG*-183G>A(rs2069709) foi monomórfico para a amostragem avaliada.

- Maior frequência do genótipo *AA* para o SNP *INFG*+874T/A (rs2430561) foi observada no grupo de indivíduos com malária, porém sem diferença significante.

- Os SNPs nos genes de citocinas *TNFA, INFG* e *IL10* e no receptor *CD28* investigados não foram associados com nenhuma ancestralidade genômica na amostra estudada.

-Os haplótipos nos genes *TNFA* e *IL10* não são influenciados para os dois com as ancestralidades europeia, africana e nativo americana para os dois grupos avaliados no estudo.

- O alelo A do TNF-308 diminui progressivamente com o aumento da proporção de ancestralidade europeia.

-Os polimorfismos de genes de citocinas e haplótipos avaliados não interferem com níveis de anticorpos da classe IgG anti-merozoítos (PvDBP, Pv-AMA-1 e Pv-MSP1-$_{19}$) para o *Plasmodium vivax*.

- Indivíduos com o genótipo GG para o SNP *CD28*-372G/A (rs35593994) tinham níveis mais baixos de anticorpos da classe IgM para a proteína iumnogênica anti ICB2-5.

- Para o SNP *CD28*+17T/C (rs3116496) os indivíduos com o genótipo CC apresentaram níveis mais elevados de IgG1 em relação aos portadores do genótipo TT e TC.

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APÊNDIDES

APÊNDICE I

Comitê de Ética em Pesquisa: Aprovação Projeto

FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO
Autarquia Estadual - Lei n.º 8899 de 27/09/94

Parecer nº 11868

COMITÊ DE ÉTICA EM PESQUISA

O projeto de pesquisa **CAAE nº 01774812.2.0000.5415** sob a responsabilidade de **Adriana Antônia da Cruz Furini**, com o título "Malária Vivax no Estado do Pará: Influência de polimorfismos no gene CD28 associados à produção de citocinas" está de acordo com a resolução do CNS 196/96 e foi **aprovado por esse CEP.**

Lembramos ao senhor(a) pesquisador(a) que, no cumprimento da Resolução 251/97, o Comitê de Ética em Pesquisa em Seres Humanos (CEP) **deverá receber relatórios semestrais sobre o andamento do Estudo**, bem como a qualquer tempo e a critério do pesquisador nos casos de relevância, além do envio dos relatos de eventos adversos, com certeza para conhecimento deste Comitê. **Salientamos ainda, a necessidade de relatório completo ao final do Estudo.**

São José do Rio Preto, 10 de abril de 2012.

Prof. Dr. Fernando Batigália
Presidente do CEP/FAMERP

Avenida Brigadeiro Faria Lima, 5416 - CEP 15090-000 - São José do Rio Preto - SP - Brasil
Tel. (17) 3201-5700 - Fax (17) 3229-1777 - *www.famerp.br*

APÊNDICE II

SEMINÁRIO LAVERAN & DEANE - 2012

Participação em Seminário

XVII Seminário Laveran & Deane sobre Malária

CPD - Mal

Certificado

Certificamos que Adriana Antônia da Cruz Furini participou, na qualidade de Estudante de Pós-Graduação do **XVII Seminário Laveran & Deane sobre Malária**, promovido pelo Centro de Pesquisa, Diagnóstico e Treinamento em Malária – CPD-Mal/Fiocruz, no período de 26 a 30 de novembro de 2012.

Itacuruçá, 30 de novembro de 2012

Cláudio Tadeu Daniel-Ribeiro
Coordenador do Seminário Laveran & Deane

Tânia Cremonini de Araújo-Jorge
Diretora do Instituto Oswaldo Cruz

Claude Pirmez
Vice-Presidente de Pesquisa e Laboratórios de Referência/Fiocruz

Patrocínio

BRASIL FAPERJ CAPES CNPq

Apoio:

IOC Instituto Oswaldo Cruz · Rio-Manguinhos · FIOCRUZ · sanofi aventis · ODEBRECHT · Fio

APÊNDICE III

Curso: Citometria de Fluxo - 2012

CCAB: Centro de Conhecimento Avançado Becton Dickinson

Certificado

Adriana Antonia da Cruz Furini

Participou do curso teórico-prático (14 horas)

"Quantificação de citocinas por Citometria de Fluxo (BD™CBA)"

25 a 27 de junho de 2013 – CCABD – São Paulo/SP

CCABD
Centro de Conhecimento Aplicado BD

BD
BD Biosciences

APÊNDICE IV

Congresso Brasileiro de Medicina Tropical (2013)

Participação em Congresso

APÊNDICE V

Congresso Brasileiro de Medicina Tropical (2013)

Resumo em Anais de Congresso

ÁREA TEMÁTICA: F) DOENÇAS POR PROTOZOÁRIOS

P-752

TÍTULO: POLIMORFISMO NO GENE IL10 INFLUENCIAM NA CARGA PARASITÁRIA EM UMA POPULAÇÂO COM MALÁRIA VIVAX NO ESTADO DO PARÁ.

AUTOR(ES): ADRIANA ANTÔNIA DA CRUZ FURINI , GUSTAVO CAPATTI CASSIANO, MARCELA PETROLINI CAPOBIANCO, FRANCIELE MAIRA MOREIRA BATISTA TOMAZ, PAMELLA CRISTINA ALVES TRINDADE, MICHELE ENCINAS, DIEGO LONGO MADI, LUCIANA MORAN CONCEIÇÃO, VALERIA DALTIBARI FRAGA, RICARDO LUIZ DANTAS MACHADO

INSTITUIÇÃO: FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO

No Brasil o *Plasmodium vivax* tem sido a espécie mais prevalente, responsável por aproximadamente 83% dos casos de malária na região Amazônica brasileira. Os polimorfismos nos genes de citocinas pró e anti-inflamatórias, codificadores de moléculas que interagem em vias moduladoras da resposta imune celular e humoral, podem influenciar na resistência ou susceptibilidade a malária, assim como nas concentrações dessas moléculas e resultado da infecção. A densidade parasitária tem sido reconhecida como importante fator no resultado da infecção pela malária, e dessa forma foi investigado se polimorfismos nesses genes estão associados com a parasitemia na malária vivax. Investigamos se polimorfismos em genes de citocinas estão associados com a parasitemia na malária vivax em pacientes provenientes do município de Goianésia do Pará no sudoeste Paraense. Foram investigados 83 pacientes com malária vivax e 50 indivíduos não-maláricos. Quatro SNPs foram analisados por PCR-RFLP nos genes *IFN-gama*, *TNF-alfa* e *IL-10*. A parasitemia foi determinada por contagem do número de parasitos por microlitro de sangue. Associação entre os genótipos e densidade parasitária foi determinada pelo teste de Mann-Whitney, com nível de significância de 0,05, utilizando o software estatístico R. Todos os SNPs foram testados em equilíbrio de Hardy-Weinberg. Foi observada associação significativa (p=0,13) apenas entre genótipo AA do SNP rs_1800872 no gene *IL10*, na posição -592 e densidade parasitária média de 6.000 mm^3. A IL-10 é uma citocina imunorregulatória do tipo 2, produzida no inicio da infecção por monócitos e mais intensamente por linfócitos. Esta proteína modula os efeitos das citocinas pro inflamatórias (IL-1, IL-6, IL-8, IL-12, IFN-γ e TNF-α) produzidas por células do tipo TH1 e por linfócitos TCD8 com regulação negativa. Os resultados obtidos sugerem a participação efetiva de genes humanos na modulação da resposta imune e sua influência sobre a carga parasitária da malária por *Plasmodium vivax*, essenciais no estabelecimento de estratégias de imunização e controle da doença, em área de transmissão ativa localizada no Estado do Pará.

APÊNDICE VI

13º Congresso Nacional de Iniciação Científica CONIC-SEMESP (2013)

Orientação de Trabalho

Certificamos que ADRIANA ANTÔNIA DA CRUZ FURINI

participou como orientador(a) do trabalho ANÁLISE ENTRE O POLIMORFISMO -238G>A NO GENE TNF-ALFA E A MALARIA VIVAX EM REGIÃO ENDÊMICA DO BRASIL.

Área do Conhecimento: CIÊNCIAS BIOLÓGICAS E SAÚDE
Subárea: FARMÁCIA
Categoria: EM ANDAMENTO

no 13º Congresso Nacional de Iniciação Científica CONIC-SEMESP, realizado nos dias 29 e 30 de novembro de 2013, na Faculdade Anhanguera de Campinas – Unidade 3, em Campinas - SP, com carga horária de duas horas na sessão de apresentação.

Realização: SEMESP

Apoio: Anhanguera

Hermes Ferreira Figueiredo
Presidente do Semesp

APÊNDICE VII

63 rd Annual Meeting – The American Society of Tropical Medicine and Hygiene (2014)

Resumo em Anais de Congresso

391

STUDY ON ASSOCIATION BETWEEN GENETIC POLYMORPHIMS OF TUMOR NECROSIS FACTOR-A, INTERLEUKIN-10, INTERFERON-I', AND MALARIA VIVAX IN BRAZIL

Adriana A. Furini[1], Gustavo C. Cassiano[2], Franciele M. Tomaz[1], Marcela P. Capobiano[2], Marinete M. Póvoa[3], Michele Encinas[4], Diego L. Madi[1], Pamella C. Trindade[1], Valeria D. Fraga[1], Luciana M. Conceição[1], Ricardo L. Machado[2]

[1]*Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, Brazil,* [2]*Universidade Estadual Paulista, São José do Rio Preto, Brazil,* [3]*Instituto Evandro Chagas, São José do Rio Preto, Brazil,* [4]*Centro Universitário de Rio Preto, São José do Rio Preto, Brazil*

Malaria is a major cause of morbidity and mortality in many tropical and subtropical countries. In brazil, the *Plasmodium vivax* has been the most prevalent species, accounting for approximately 83% of malaria cases in the brazilian amazon region. Despite of the clinical descriptions of the disease caused by *P vivax* is well described, standards regarding humoral and cellular immune response, as well as the pattern of cytokines are scarce and not fully understood. Polymorphisms in genes cd28, ifny, tnfa and il10, that are encoder molecules that interact in modulating pathways of the cellular and humoral immune response, may influence the resistance or susceptibility to malaria. We had analyzed 90 blood samples from patients with vivax malaria diagnosed by molecular and non-molecular techniques and 51 from non-malarial and all from goianésia of pará city, pará state, brazil. Polymorphisms in genes IL10 (-819 c>t, -592 c>a), ifnγ (-183 g>t), tnfα (-238g>a), and cd28 (-372g>a, +17t>c) were analyzed by PCR-RFLP. All subjects were genotyped with 48 ancestry informative insertion-deletion polymorphisms to determine the proportion of African, European and Amerindian ancestry to avoid bias due to differences in ancestry contributions in malaria and non-malaria groups. We used the fisher exact test to measure association between genotypes and malaria infection. All polymorphisms tested were in hardy-weinberg equilibrium. The African, European andl native American admixture did not differ among cases and controls. No significant association was found between the polymorphisms tested and vivax malaria and non-malarial individuals. P-values in co-dominant, dominant and recessive models were also calculated and no significant association was found. These findings make us to believe that the analyzed polymorphisms are not associated with susceptibility or resistance with vivax malaria in the studied population.the results will be finalized by june 2016.

APÊNDICE VIII

XXXIX Congress of the Brazilian Society of Immunology (2014)

Participação em Congresso


IMMUNO BÚZIOS 2014
XXXIX Congress of the Brazilian Society of Immunology
VII ESCI - Extra Section of Clinical Immunology

October 18 - 22, 2014
Atlântico Búzios Hotel
Búzios - RJ, Brazil

Organization
SBI
Sociedade Brasileira de Imunologia

Certificate

ADRIANA ANTONIA DA CRUZ FURINI
has participated in the
XXXIX Congress of the Brazilian Society of Immunology 2014
from 18 to 22 October 2014, Búzios, Brazil

Búzios - RJ, Brazil, October 22nd, 2014.

João P. B. Viola
President

APÊNDICE IX

XXXIX Congress of the Brazilian Society of Immunology

Apresentação de trabalho: pôster

IMMUNO BÚZIOS 2014
XXXIX Congress of the Brazilian Society of Immunology
VII ESCI - Extra Section of Clinical Immunology

October 18 - 22, 2014
Atlântico Búzios Hotel
Búzios - RJ, Brazil

Organization
SBI
Sociedade Brasileira de Imunologia

Certificate

We hereby certify that the scientific papers entitled

PLASMODIUM VIVAX MALARIA IN THE PARA STATE: ASSOCIATION OF TNF-ALFA AND IL-10 POLYMORPHISMS WITH ANTIBODIES AGAINST PVAMA-1

with the authors

ADRIANA ANTÔNIA DA CRUZ FURINI; GUSTAVO CAPATTI CASSIANO; FRANCIELE MAIRA TOMAZ; MARCELA PETROLINI CAPOBIANCO; PAMELLA CRISTINA ALVES TRINDADE; MICHELE ENCINAS; DIEGO LONGO MADI; LUCIANA MORAN CONCEIÇÃO; VALÉRIA DALTIBARI FRAGA; SIDNEY EMANUEL BATISTA DOS SANTOS; MARINETE MARINS POVOA; RICARDO LUIZ DANTAS MACHADO.

was presented as **POSTER**

in the **XXXIX Congress of the Brazilian Society of Immunology 2014**

Búzios - RJ, Brazil, October 22nd, 2014.

João P. B. Viola
President

APÊNDICE X

Med Trop 2015 – 51º Congresso da Sociedade Brasileira de Medicina Tropical

Apresentação de trabalho: pôster

MedTrop 2015
51º Congresso da Sociedade Brasileira de Medicina Tropical

14 a 17 de junho de 2015
Centro de Eventos do Ceará
Fortaleza/CE - Brasil

Doenças Tropicais: do ensino e pesquisa aos serviços de saúde

CERTIFICADO

Certificamos que o trabalho

OS POLIMORFISMOS NOS GENES DAS CITOCINAS TNF-ALFA, IL-10 E IFN-GAMA PODEM INFLUENCIAR NOS NÍVEIS DE ANTICORPOS CONTRA MSP1-19 NOS PACIENTES COM MALARIA VIVAX?

Tendo como autores: Adriana Antônia da Cruz Furini, Gustavo Capatti Cassiano, Marcela Petrolini Capobianco, Luciana Moran Conceição, Valéria Daltibari Fraga, Maristela Gomes da Cunha, Ricardo Luiz Dantas Machado,
foi apresentado no 51º Congresso da Sociedade Brasileira de Medicina Tropical - MEDTROP,
como Pôster na categoria Doenças por protozoários.

Fortaleza, 29 de maio de 2015.

Mitermayer Galvão dos Reis
Presidente da SBMT

Ivo Castelo Branco Coelho
Presidente do Congresso

Luciano Pamplona de Góes Cavalcanti
Presidente da Comissão Científica

Para verificar a autenticidade deste certificado, acesse o link http://medtrop2015.gnius.com.br/certificado e informe o código: 07025411

APÊNDICE XI

Programa de Pós Graduação em Genética – UNESP/IBILCE

Discussão de Seminários: Genética de Parasitos

MS - SECRETARIA DE VIGILÂNCIA EM SAÚDE
INSTITUTO EVANDRO CHAGAS
Rodovia BR-316 Km 7 s/n - Levilândia - 67030-000
Ananindeua - Pará - Brasil
contato@iec.pa.gov.br

Belém, 15 de julho de 2015

A quem possa interessar,

Declaro para os devidos fins que a PROFA MsC ADRIANA ANTÔNIA DA CRUZ FURINI ministrou aula sobre "ASPECTOS DA RESPOSTA IMUNE HUMANA EM DOENÇAS CAUSADAS POR PROTOZOÁRIOS" e participou da discussão de seminários na disciplina **Genética de Parasitos** ministrada no Programa de Pós-Graduação em Genética na UNESP/IBILCE de São José do Rio Preto.

Outrossim, informo-vos que a disciplina foi ministrada no período de 22/05/2015 a 29/05/2015 com carga horária de 60 horas.

Sem mais, subescrevo-me atenciosamente.

Ricardo Luiz Dantas Machado

Prof. Doutor e Livre-Docente em Parasitologia

Instituto Evandro Chagas/SVS/MS

APÊNDICE XII

FIOCRUZ – RIO DE JANEIRO

Treinamento em Citometria de Fluxo

FUNDAÇÃO OSWALDO CRUZ
Instituto Oswaldo Cruz

Declaração

Declaro para os devidos fins que a professora Adriana Antonia da Cruz fez um treinamento no Laboratório de Imunoparasitologia do Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, no período de 6 a 10 de julho de 2015. O treinamento foi em citometria de fluxo para determinar a concentração de citocinas em amostras de plasma.

Atenciosamente,

Dra Joseli de Oliveira Ferreira
Pesquisadora Titular
Laboratório de Imunoparasitologia
Instituto Oswaldo Cruz - Fiocruz

APÊNDICE XIII

XIV Reunião Nacional de Pesquisa em Malária (2015)

Participação em Congresso

XIV REUNIÃO NACIONAL DE PESQUISA EM MALÁRIA

CERTIFICADO

30 DE SETEMBRO A 1 DE OUTUBRO DE 2015

Certificamos que

Adriana Antonia da Cruz Furini

participou da XIV Reunião Nacional de Pesquisa em Malária realizada de 30 de Setembro a 3 de Outubro de 2015 no Centro de Convenções Rebouças, São Paulo – SP.

FÁBIO TRINDADE MARANHÃO COSTA (UNICAMP)
PRESIDENTE DA COMISSÃO CIENTÍFICA

MARCELO URBANO FERREIRA (USP)
PRESIDENTE DA COMISSÃO ORGANIZADORA

APÊNDICE XIV

XIV Reunião Nacional de Pesquisa em Malária (2015)

Apresentação de trabalho: pôster

XIV REUNIÃO NACIONAL DE PESQUISA EM MALÁRIA

30 DE SETEMBRO A 1 DE OUTUBRO DE 2015

CERTIFICADO

Certificamos que o Trabalho nº **PT.006** com o título: "**POLIMORFISMOS EM GENES DE CITOCINAS E A PRODUÇÃO DE ANTICORPOS CONTRA PVDBP, AMA-1 E MSP119 EM INDIVÍDUOS NATURALMENTE INFECTADOS COM PLASMODIUM VIVAX EM UMA ÁREA ENDÊMICA DA AMAZÔNIA BRASILEIRA.**", de autoria de *Furini AAC, Cassiano GC, Capobianco MP, Póvoa MM, Carvalho LH, Kano FS, Soares I, Cunha MG, Machado RLD* foi apresentado como **pôster** na XIV Reunião Nacional de Pesquisa em Malária realizada de 30 de Setembro a 3 de Outubro de 2015 no Centro de Convenções Rebouças, São Paulo – SP.

FÁBIO TRINDADE MARANHÃO COSTA (UNICAMP)
PRESIDENTE DA COMISSÃO CIENTÍFICA

MARCELO URBANO FERREIRA (USP)
PRESIDENTE DA COMISSÃO ORGANIZADORA

APÊNDICE XV

Artigos publicados no Doutorado

1.Furini AAC, Lima TAM, Rodrigues LV, Fachina F, Galão EG, Santin MS, Rossit ARB, Machado RLD. Prevalence of intestinal parasitosis in a population of children of a daycare in Brazil. Parasitaria 2015; 21 (1):1-5.

2. Furini AAC, Guimaraes PM, Atique Netto HA, Castro KF, Silva BTOS, Atique, TSC, Chiaparini J, Ramos MPSCM, Martins EA, Nardo CDD. Análise epidemiológica, identificação e perfil de susceptibilidade a antimicrobianos isolados de cães com infecção do trato urinário. Acta Veterinaria Brasilica (UFERSA) 2014;7:288-93.

3.Furini AAC, Prates DC, Pezzini AP, Rabecini Junior LC, Regino BB, Schiesari AJ, Machado RLD. Epidemiologia da coinfecção por HIV/HCV em um hospital escola de Catanduva, São Paulo - Brasil. Revista do Instituto Adolfo Lutz 2014; 73: 106-12.

4.Furini AAC, Guimarães PM, Silva FSE, Lopes MASM, Castro KF, Atique, TSC, Atique Netto H. Estudo da variação neutropênica pelo sulfato de vincristina em cães com tvt tratados no Hospital Veterinário do Noroeste paulista. Arquivos de Ciências Veterinárias e Zoologia da UNIPAR 2014; 17: 5-9.

5.Furini AAC, Lima TAM, Faitarone NC, Verona JP, Silva LAM, Santos SS, Reis AG, Guimarães PM, Atique TSC . Atenção Farmacêutica nas Interações

Medicamentosas e Indicadores de Prescrição em uma Unidade Básica de Saúde. Arquivos de Ciências da Saúde 2014; 21: 99-06.

6.Furini AAC, Lima TAM, Rocha WM, Teixeira BCA, Martins AA, Feltrin J, Seara MO, Gonçalves RR, Atique TSC. Acompanhamento farmacoterapêutico em paciente idoso: relato de interações medicamentosas. Rev Eletr Pesq UNIRP – Universitas 2004; 4:110-121.

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Immunogenetic markers associated with a naturally acquired humoral immune response against an N-terminal antigen of *Plasmodium vivax* merozoite surface protein 1 (PvMSP-1)

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Abstract

Background: Humoral immune responses against proteins of asexual blood-stage malaria parasites have been associated with clinical immunity. However, variations in the antibody-driven responses may be associated with a genetic component of the human host. The objective of the present study was to evaluate the influence of co-stimulatory molecule gene polymorphisms of the immune system on the magnitude of the humoral immune response against a *Plasmodium vivax* vaccine candidate antigen.

Methods: Polymorphisms in the *CD28*, *CTLA4*, *ICOS*, *CD40*, *CD86* and *BLYS* genes of 178 subjects infected with *P. vivax* in an endemic area of the Brazilian Amazon were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The levels of IgM, total IgG and IgG subclasses specific for ICB2-5, i.e., the N-terminal portion of *P. vivax* merozoite surface protein 1 (PvMSP-1), were determined by enzyme-linked immuno assay. The associations between the polymorphisms and the antibody response were assessed by means of logistic regression models.

Results: After correcting for multiple testing, the IgG1 levels were significantly higher in individuals recessive for the single nucleotide polymorphism rs3116496 in *CD28* ($p = 0.00004$). Furthermore, the interaction between *CD28* rs35593994 and *BLYS* rs9514828 had an influence on the IgM levels ($p = 0.0009$).

Conclusions: The results of the present study support the hypothesis that polymorphisms in the genes of co-stimulatory components of the immune system can contribute to a natural antibody-driven response against *P. vivax* antigens.

Keywords: *Plasmodium vivax*, MSP-1, ICB2-5, Immunogenetics, Antibodies

Background

According to the World Health Organization, there were an estimated 200 million cases of malaria in 2014 and an estimated 584,000 resulting deaths worldwide [1]. Among the five species that can cause malaria in humans, *Plasmodium vivax* is the most widely distributed, accounting for most of the cases of malaria in South and Southeast Asia, Latin America and Oceania; there are an estimated 2.5 billion people living in areas at risk of transmission of the disease [1, 2]. Furthermore, although vivax malaria

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has not been considered life-threatening for a long time, reports of severe cases associated with *P. vivax* have been increasingly numerous [3].

The blood stage of the *Plasmodium* lifecycle is responsible for the pathology associated with malaria. In this stage, merozoites released from schizont-infected erythrocytes invade non-infected erythrocytes, resulting in their destruction and the release of more merozoites into the bloodstream. During this brief extracellular period, these free merozoites are exposed to the host immune system, and proteins that are critical for parasite invasion are, therefore, important targets for the development of malaria vaccines. Merozoite surface proteins (MSPs) are among the most studied, especially MSP-1, which is necessary for merozoite attachment to erythrocytes [4] and normal parasite development [5].

The most widely accepted structure of the *P. vivax* merozoite surface protein 1 (PvMSP-1) gene indicates that it consists of six polymorphic blocks (blocks 2, 4, 6, 8, 10 and 12) flanked by seven conserved blocks (blocks 1, 3, 5, 7, 9, 11 and 13) [6]. By analysing the primary structure of PvMSP-1, several seroepidemiological studies have been performed to evaluate its immunogenic potential [7–11]. Although the C-terminal portion of the protein (PvMSP-1_{19}) is the most immunogenic [7, 9, 12], a number of studies showed high prevalence of IgG against N-terminal PvMSP-1 in individuals exposed to *P. vivax* [9, 10, 13, 14]. Furthermore, specific IgG3 antibodies against the N-terminal portion of PvMSP-1 have been associated with clinical protection in two riverine communities of the Brazilian Amazon [9, 11], similar to that observed in *P. falciparum* infection, where persistence of antibodies IgG3 against N-terminal of MSP-1 was related with prolongation time without malaria [15]. In fact, antibodies specific for the asexual blood stage are thought to be involved in clinical protection against malaria vivax. Longitudinal cohort studies have shown correlations between magnitude of antibody responses to *P. vivax* merozoite antigens and protection from malaria [11, 16–18]. Due to the inability in maintaining *P. vivax* in continuous in vitro culture, it is difficult to define the role of antibodies to this species, but few evidences support that it may be related to inhibition of merozoite invasion [19, 20]. Furthermore, complement and FcR mediated mechanisms seem to be important in antibody-mediated protection [21].

The development of an adequate immune response depends on the fine regulation of lymphocyte activation. For this, in general, lymphocytes require two activation signals. The first signal is antigen-specific, whereas the second signal, called co-stimulation, is generated by the interaction between the surface molecules of T cells and those of antigen-presenting cells, including B cells. The interaction between CD28 and its ligands, CD80 and CD86, provides the strongest costimulatory signal for T-cell proliferation, whereas CTLA-4 is a negative regulator that plays a key role in T cell homeostasis and in central tolerance [22]. Another member of the CD28 family, Inducible co-stimulator (ICOS), is an important immune regulatory molecule that participates in T-cell activation and T-cell dependent B-cell responses [23, 24]. CD40 is presented on the surface of B-cells and the CD40-CD40L interaction is the major costimulatory signal for B cells to mount a humoral response [25]. B lymphocyte stimulator (BLYS) is produced mainly by innate immune cells and is needed to provide signals for B cell survival and proliferation [26]. Considering the importance of these molecules in development of immune response and because there are currently multiple lines of evidence showing that the genes involved in the immune response can influence antibody production during malaria infection [27–34], the authors hypothesised that polymorphisms in the genes of the co-stimulatory molecules CD28, CTLA-4, ICOS, CD86, CD40 and BLYS are involved in the magnitude of the naturally acquired antibody-driven response against N-terminal PvMSP-1 in individuals infected with *P. vivax* in the Brazilian Amazon.

Methods

Study area and subjects

The present study was conducted in the municipality of Goianésia do Pará, Pará state, Brazil, which is a constituent of the Brazilian Amazon region, where *P. vivax* is responsible for more than 80 % of all malaria cases [35]. Details of the study area have been described elsewhere [36]. Cross-sectional surveys conducted from February 2011 to August 2012 were used to recruit 178 subjects (125 men and 53 women) presenting with classic symptoms of malaria, who sought treatment at the medical service of the municipality; the subjects had an average age of 29.8 years (varying from 14 to 68 years). Exclusion criteria included children under 10 years old, pregnancy, related individuals and anti-malarial treatment within the previous seven days. Diagnosis was performed by microscopy (thick film), and infection with *P. vivax* was subsequently confirmed by nested polymerase chain reaction (PCR) [37]. The geometric mean of parasiatemia was 718.4 parasites/mm^3 (95 % CI 487.2–1059.2). All patients with microscopically confirmed infections were given standard treatment of 25 mg/kg of chloroquine in 3 days plus 0.5 mg/kg of primaquine during seven days. All participants were submitted to a questionnaire to obtain epidemiological information. The length of residence in the municipality varied between one month and

37 years (median of 5 years), and 91.9 % of the individuals had previously contracted malaria. Samples from 40 malaria-naive individuals living in a non-endemic area (São José do Rio Preto, Brazil), who had never been to areas of malaria transmission, were used as controls.

Peripheral venous blood (~10 mL) was collected in EDTA tubes, and plasma samples were obtained by centrifugation and stored at −20 °C. The protocol of the present study was approved by the Research Ethics Committee of the Medical School of São José do Rio Preto (CEP/FAMERP no. 4599/2011) and by the health authorities of the municipality of Goianésia do Pará. All participants or their legal guardians signed an informed consent form.

Genotyping

DNA was extracted from peripheral blood samples with an Easy-DNA™ extraction kit (Invitrogen, California, USA). All single nucleotide polymorphisms (SNPs) were genotyped by PCR-restriction fragment length polymorphism (RFLP). The SNPs *−372G > A* and *+17T > C* in *CD28* (rs35593994 and rs3116496, respectively), *−318C > T* and *+49A > G* in *CTLA4* (rs5742909 and rs231775, respectively), *+1564T > C* in *ICOS* (rs4404254), *+1057G > A* in *CD86* (rs1129055), *−1C > T* in *CD40* (rs1883832), and *−871C > T* in *BLYS* (rs9514828) were genotyped according to previously described protocols [36, 38, 39]. Primer sequences, restriction enzymes, and the restriction fragments obtained of each SNP are presented in Additional file 1. PCR for the identification of *−1722T > C* and *−1577G > A* in *CTLA4* (rs733618 and rs11571316, respectively) was performed with a final sample volume of 25 µL containing 1X Buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.6 pmol of each primer, and 0.5 U Platinum Taq DNA Polymerase (Invitrogen, São Paulo, Brazil). The primers 5′ CTTCATGCCGTTTCCAACTT 3′ and 5′ CCTTTTCTGACCTGCCTGTT 3′ were used for the *−1722T > C* genotyping, whereas 5′ CTTCAT GCCGTTTCCAACTT 3′ and 5′ ATCTCCTCCAGGAA GCCTCTT 3′ were used to identify *−1577G > A*. Amplifications were performed under the following conditions: first step of 5 min at 94 °C, 35 cycles for 30 s at 94 °C, 30 s at 52 °C and 1 min at 72 °C, and a final step for 10 min at 72 °C. The PCR products of *−1722T > C* and *−1577G > A* were digested with the enzymes *Bbv*I and *Mbo*II (Fermentas, Vilnius, Lithuania), respectively. Electrophoresis was performed in 2.5 % agarose and stained with GelRed™ (Biotium, Hayward, USA) with the exception of the rs5742909 and rs1883832 SNPs, which were performed in 12.5 % polyacrylamide gel after staining with ethidium bromide, and visualized in a UV transilluminator.

Antigen and antibody determination

The glutathione-S-transferase (GST)-tagged recombinant ICB2-5 protein corresponds to the amino acids 170-675 of the N-terminal portion of MSP-1 from the Belém strain of *P. vivax*. The ICB2-5-GST fusion protein was purified on a glutathione-Sepharose 4B column (Amersham Pharmacia), and the protein content was assessed with the Bio-Rad Protein Assay Kit I (Bio-Rad Laboratories, Inc.) [7]. The levels of IgM and IgG subclasses specific for ICB2-5 were measured as described previously with some modifications [7]. The plates were coated with GST-tagged ICB2-5 and GST alone, and all tests were done in duplicate. Briefly, 50 µL of ICB2-5-GST or GST alone (as a control) was coated on ELISA plates at 4 µg/mL (Costar, Corning Inc., New York, USA) and incubated overnight at 4 °C. After washing and blocking the plates with 0.05 % bovine serum albumin-PBS, 50 µL of plasma diluted 1/100 was added to the wells in duplicate and incubated for 1 h at 37 °C. Then, 50 µL of peroxidase-conjugated anti-human IgG or IgM antibodies (KPL, Maryland, USA) diluted 1/1000 were added to each well for the detection of total IgG or IgM, respectively. IgG subclasses were detected using mouse monoclonal antibodies specific for each isotype (Sigma, Missouri, USA), diluted according to the tested subclass (1/3000 for IgG1 clone HP6001; and IgG3 clone HP6050, 1/2500 for IgG2 clone HP6014, and 1/5000 for IgG4 clone HP6025). The immobilized monoclonal antibodies were detected with peroxidase-conjugated anti-mouse antibodies for 1 h at 37 °C (Sigma, Missouri, USA). Subsequently, the plates were washed and developed in the dark with 50 µL TMB substrate diluted 1/50 with 0.1 M phosphate-citrate buffer, pH 5.0, containing 0.03 % H_2O_2. The reaction was interrupted after 10 min by adding 50 µL of 2 N H_2SO_4 to each well and read at 450 nm. The determination of positivity for anti ICB2-5 was calculated as described previously, with some modifications [9]. Brief, firstly the average OD was calculated for each individual, and serum was considered positive if GST-tagged ICB2-5 OD was equal to or greater than cut off for this same protein, measured with sera of individuals who never had a past history of malaria. To confirm the positivity of a serum for anti ICB2-5, the average of GST-tagged ICB2-5 OD was calculated to exclude reactivity against GST. For each serum, the GST-cut off was calculated as average of GST OD plus twice the standard deviations (SD). The positivity for anti ICB2-5 was determined when average of GST-tagged ICB2-5 OD was equal to or greater than its GST-cut off. The results are expressed as the reactivity index (RI), which was calculated by dividing the OD of the sample by the cut-off value. Only the samples that were positive for total IgG (RI > 1) were tested for IgG subclasses.

Statistical analysis

The associations between the SNP genotypes and the antibody response were analysed by means of a logistic regression model, controlling for potential confounders, such as age and gender. Different genetic models (codominant, dominant, recessive and log-additive) were tested with the 'SNPSassoc' package [40] implemented for the R statistical software (version 3.1.1). A Bonferroni correction was used to adjust for multiple testing, and the significance was set at $p < 0.005$ (0.05/10). The sample size was calculated using GWAPower software [41]. The current sample size (n = 178) had 80 % power to detect a variant with about 6 % heritability. Since only 90 samples were assessed for IgG subclasses, the power to detect the same heritability was about 42 %. Genotypic deviations from the Hardy–Weinberg equilibrium (HWE) were evaluated by using the exact test described by Wigginton et al. [42]. Statistically significant differences between the means of continuous variables were assessed with one-way analysis of variance (ANOVA). Correlation coefficients of the antibody levels were estimated with the Pearson correlation. Interactions between SNP pairs were also assessed with the 'SNPassoc' package, which determines the effects of interaction by means of log-likelihood ratio tests (LRTs).

Haplotype blocks were determined with Haploview 4.2 using the solid spine of linkage disequilibrium (LD) method [43], and the degree of LD between SNPs was estimated with the D' parameter. The R package 'haplo.stats' [44] was used for association tests between haplotypes and antibody levels by means of the 'haplo.glm' function, which performs a regression that estimates the effect of each haplotype compared to a reference haplotype by means of a general linear model.

Results

Serological data and single-marker associations

Of the 178 subjects analysed, all infected with *P. vivax*, 90 (50.6 %) exhibited IgG (RI > 1), whereas 53 (29.8 %) exhibited IgM specific for ICB2-5. The antibody levels were higher for IgG1 (mean = 1.08) compared to those of the other subclasses (means of 0.91, 0.79 and 0.79 for IgG2, IgG3 and IgG4, respectively) ($p < 0.001$, ANOVA). The highest correlations between levels of IgG subclasses were found for IgG1 and IgG2 (r = 0.48) and IgG1 and IgG3 (r = 0.43) (Additional file 2). The levels of ICB2-5-specific antibodies were not influenced by previous exposure to malaria (age, length of residence in the studied area, and previous episodes of malaria) (Additional file 3), except for IgG2, which exhibited higher levels in individuals who reported to have had less than five previous episodes of malaria, compared to those who reported to have had more than five previous episodes [mean (confidence interval): 0.97 (0.84–1.10) vs. 0.88 (0.76–0.99), $p = 0.01$, ANOVA].

The allelic and genotype frequencies of all the analysed SNPs are listed in Table 1 and Additional file 4. The allele frequencies of these SNPs were previously presented in a larger set of samples (with exception of SNPs rs733618 and rs11571316) [45]. The success rate of SNP genotyping was 100 % for eight of the studied SNPs, whereas rs733618 and rs11571316 were successfully genotyped in 88.2 and 82 % of the samples, respectively, and no deviation from HWE was observed (all p value >0.1). A summary of the statistics for all the evaluated SNPs is listed in Table 1 and Additional file 4. There is not significant association between the polymorphisms and parasitaemia (all p value >0.07, Kruskal–Wallis test, data not show). Three SNPs were significantly associated with the humoral immune response against *P. vivax* ICB2-5. IgM levels were associated with rs35593994 in *CD28*; individuals with the *GG* genotype had lower antibody levels (mean 0.67 vs. 0.88, $p = 0.03$). Based on a recessive model, individuals with a *CC* genotype for rs3116496 in *CD28* exhibited higher IgG1 levels with respect to the other genotypes (mean 3.13 vs. 1.04, $p = 0.00004$). Individuals with a *TC* genotype for rs733618 in *CTLA4* exhibited higher IgG2 levels with respect to homozygous individuals (mean 1.18 vs. 0.87, $p = 0.04$). However, after correction for multiple testing, only association that remained significant was the one with *CD28* (re3116496).

Linkage disequilibrium analysis and haplotype association

Linkage disequilibrium analysis was performed for all possible pairwise combinations of seven SNPs that were analysed across 255 kb in the 2q33 chromosomal region, which encompasses *CD28*, *CTLA4* and *ICOS*. Two haplotype blocks were defined using the criterion described by Barret et al. [43] (Fig. 1). Three haplotypes of block 1, which contains rs35593994 and rs3116496 in *CD28*, were built, with frequencies varying between 18.5 and 52.2 %, and for block 2, which contains rs733618, rs11571316 and rs5742909 in the promoter region of *CTLA4*, four haplotypes were built, with frequencies varying between 6.3 and 51.8 %. The effects of each haplotype on the antibody levels were estimated and are shown in Table 2. The AT haplotype in block 1 was significantly associated with a 20 % increase in IgM levels ($p = 0.027$) with respect to the reference haplotype (specifically, the most frequent haplotype, i.e., CT). However, no association remained significant after correction for multiple testing.

Interaction analysis

Using the R project package 'SNPassoc' [40], the study explored all pairwise SNP−SNP interactions under the codominant model. These interactions are represented

Table 1 Levels of *Plasmodium vivax* ICB2-5-specific IgM, IgG and IgG subclasses with respect to the analysed genotypes from individuals infected with *P. vivax*

SNP	Gene	Genotypes	Genotypes (n^a)	Genotypes (n^b)	IgG (RI)	IgG1 (RI)	IgG2 (RI)	IgG3 (RI)	IgG4 (RI)	IgM (RI)
rs35593994	*CD28*	GG/GA/AA	86/80/12	42/39/8	1.02/1.03/1.11	1.11/1.10/0.89	0.92/0.93/0.80	0.76/0.86/0.64	0.77/0.83/0.74	0.66/0.86/1.13[c]
rs3116496	*CD28*	TT/TC/CC	118/54/6	60/27/2	1.04/1.02/0.91	1.06/0.98/3.13[d]	0.91/0.92/0.95	0.82/0.72/0.75	0.82/0.75/0.67	0.83/0.70/0.65
rs733618	*CTLA4*	TT/TC/CC	135/20/2	72/7/1	1.06/0.89/1.05	1.11/0.97/0.81	0.87/1.18/0.64[e]	0.80/0.66/0.57	0.80/0.68/0.62	0.82/0.72/1.25
rs11571316	*CTLA4*	GG/GA/AA	59/75/12	29/39/4	1.04/1.03/0.97	1.17/1.10/0.84	0.96/0.95/0.68	0.74/0.86/0.56	0.73/0.82/0.66	0.81/0.75/0.79
rs5742909	*CTLA4*	CC/CT/TT	151/27/0	75/14/0	1.15/1.01/–	1.04/1.29/–	0.91/0.93/–	0.80/0.76/–	0.80/0.78/–	0.79/0.78/–
rs231775	*CTLA4*	AA/AG/GG	75/84/19	40/41/8	1.07/1.00/1.01	0.96/1.22/0.98	0.87/0.98/0.82	0.78/0.82/0.70	0.79/0.79/0.81	0.79/0.75/0.89
rs4675378	*ICOS*	TT/TC/CC	77/73/27	35/38/16	1.00/1.05/1.09	1.08/1.12/0.99	0.86/0.94/0.96	0.73/0.90/0.67	0.80/0.78/0.80	0.80/0.73/0.85
rs1129055	*CD86*	GG/GA/AA	110/60/8	53/31/5	1.00/1.08/1.15	1.08/1.09/1.06	0.93/0.90/0.93	0.78/0.82/0.70	0.82/0.76/0.70	0.81/0.73/0.90
rs1883832	*CD40*	CC/CT/TT	135/37/6	65/20/4	1.01/1.06/1.25	1.11/1.03/0.86	0.92/0.95/0.68	0.81/0.77/0.65	0.81/0.75/0.79	0.80/0.69/1.13
rs9514828	*BLYS*	CC/CT/TT	103/62/13	48/35/6	0.99/1.07/1.15	0.99/1.23/0.98	0.91/0.94/0.83	0.82/0.69/1.11	0.76/0.81/0.92	0.73/0.83/0.95

[a] Number of individuals evaluated for ICB2-5-specific IgM and total IgG ($n = 178$)

[b] Number of individuals evaluated for ICB2-5-specific IgG subclasses ($n = 90$)

[c] Significant according to the dominant model ($p = 0.033$)

[d] Significant according to the recessive model ($p = 0.00004$)

[e] Significant according to the overdominant model ($p = 0.042$)

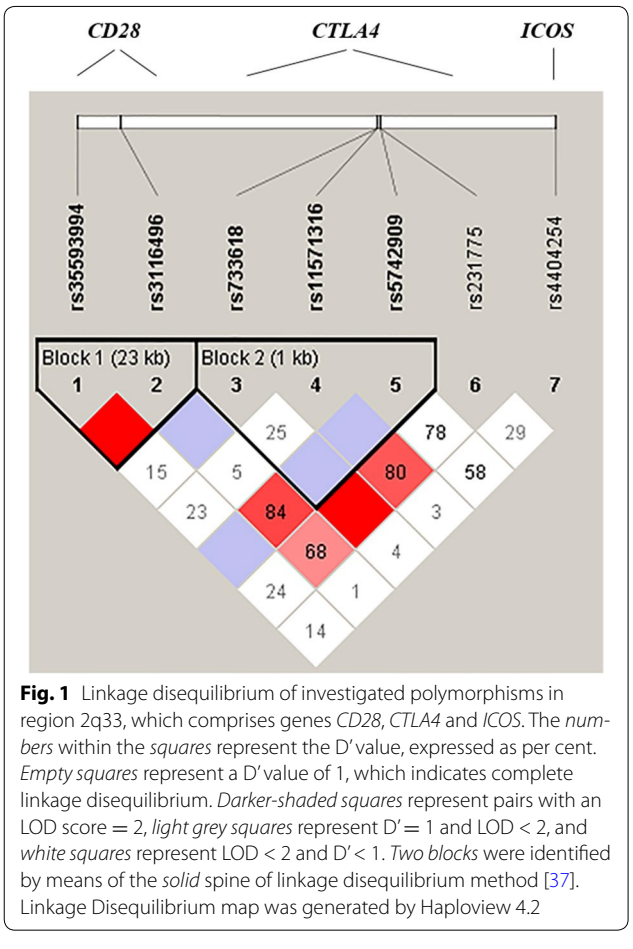

Fig. 1 Linkage disequilibrium of investigated polymorphisms in region 2q33, which comprises genes *CD28*, *CTLA4* and *ICOS*. The *numbers* within the *squares* represent the D′ value, expressed as per cent. *Empty squares* represent a D′ value of 1, which indicates complete linkage disequilibrium. *Darker-shaded squares* represent pairs with an LOD score = 2, *light grey squares* represent D′ = 1 and LOD < 2, and *white squares* represent LOD < 2 and D′ < 1. *Two blocks* were identified by means of the *solid* spine of linkage disequilibrium method [37]. Linkage Disequilibrium map was generated by Haploview 4.2

graphically in Fig. 2, in which the upper part contains the *p* values for the interaction LRT. Although several significant associations involving rs3116496 in *CD28* and other SNPs (rs1129055, rs5742909 and rs231775) with IgG1 levels were observed, the level of significance was below that found for rs3116496 individually (all *p* values >0.0001). However, the interaction involving rs35593994 in *CD28* with rs9514828 in *BLYS* was associated with the IgM levels ($p = 0.0009$). A regression analysis was performed to confirm this interaction, and the results of significant associations involving the above two SNPs are listed in Table 3.

Discussion

The main strategies used for malaria control are based on prompt diagnosis and treatment and on vector control. However, new resistant parasite strains arise as new drugs are applied, and vector control is also encountering great challenges due to the growing resistance to insecticides, thus justifying research on the development of a vaccine that is effective against malaria. Characterisation of the naturally acquired immune response in different populations is a useful tool for the identification of molecules that can be targeted by anti-malarial vaccines.

The present study was to evaluate the naturally acquired immune response against the N-terminal portion of PvMSP-1. Although the C-terminal portion ($MSP\text{-}1_{19}$) is considered to be the most immunogenic region of the protein [7, 9, 12], there is evidence suggesting that antibodies targeting the N-terminal portion

Table 2 Haplotype frequencies and their association with levels of ICB2-5-specific antibodies

Block	Haplotype	Frequency	IgG		IgG1		IgG2		IgG3		IgG4		IgM	
			Δ%	*p*	Δ%	*p*	Δ%	*p*	Δ%	*p*	Δ%	*p*	Δ%	*p*
Block 1	G-T	0.522	Reference											
	A-T	0.292	2 (−9; 13)	0.72	−3 (−27; 21)	0.80	−3 (−16; 9)	0.61	0 (−15; 14)	0.95	0 (−10; 10)	0.99	20 (2; 38)	*0.027*
	G-C	0.185	−3 (−16; 9)	0.59	21 (−9; 51)	0.17	0 (−15; 16)	0.96	−8 (−27; 10)	0.38	−7 (−19; 5)	0.26	−6 (−27; 15)	0.58
Block 2	T-G-C	0.518	Reference											
	T-A-C	0.330	−2 (−14; 10)	0.72	−4 (−32; 25)	0.81	−9 (−23; 5)	0.21	2 (−16; 19)	0.85	3 (−9; 16)	0.59	−7 (−28; 13)	0.48
	T-G-T	0.076	12 (−7; 31)	0.22	49 (−23; 121)	0.18	3 (−21; 27)	0.83	−4 (−34; 25)	0.77	−3 (−23; 17)	0.77	−3 (−34; 27)	0.84
	C-G-C	0.063	−17 (−38; 11)	0.27	−10 (−70; 51)	0.75	−14 (−44; 17)	0.38	−14 (−52; 23)	0.45	−11 (−36; 14)	0.38	−23 (−54; 9)	0.16

The effects of each haplotype are relative to the most frequent haplotype, which was used as a reference. Δ% indicates changes relative to the antibody levels compared to reference haplotypes, with 95 % confidence intervals. Block 1 refers to the SNPs rs35593994 and rs3116496, and block 2 refers to rs733618, rs11571316 and rs5742909

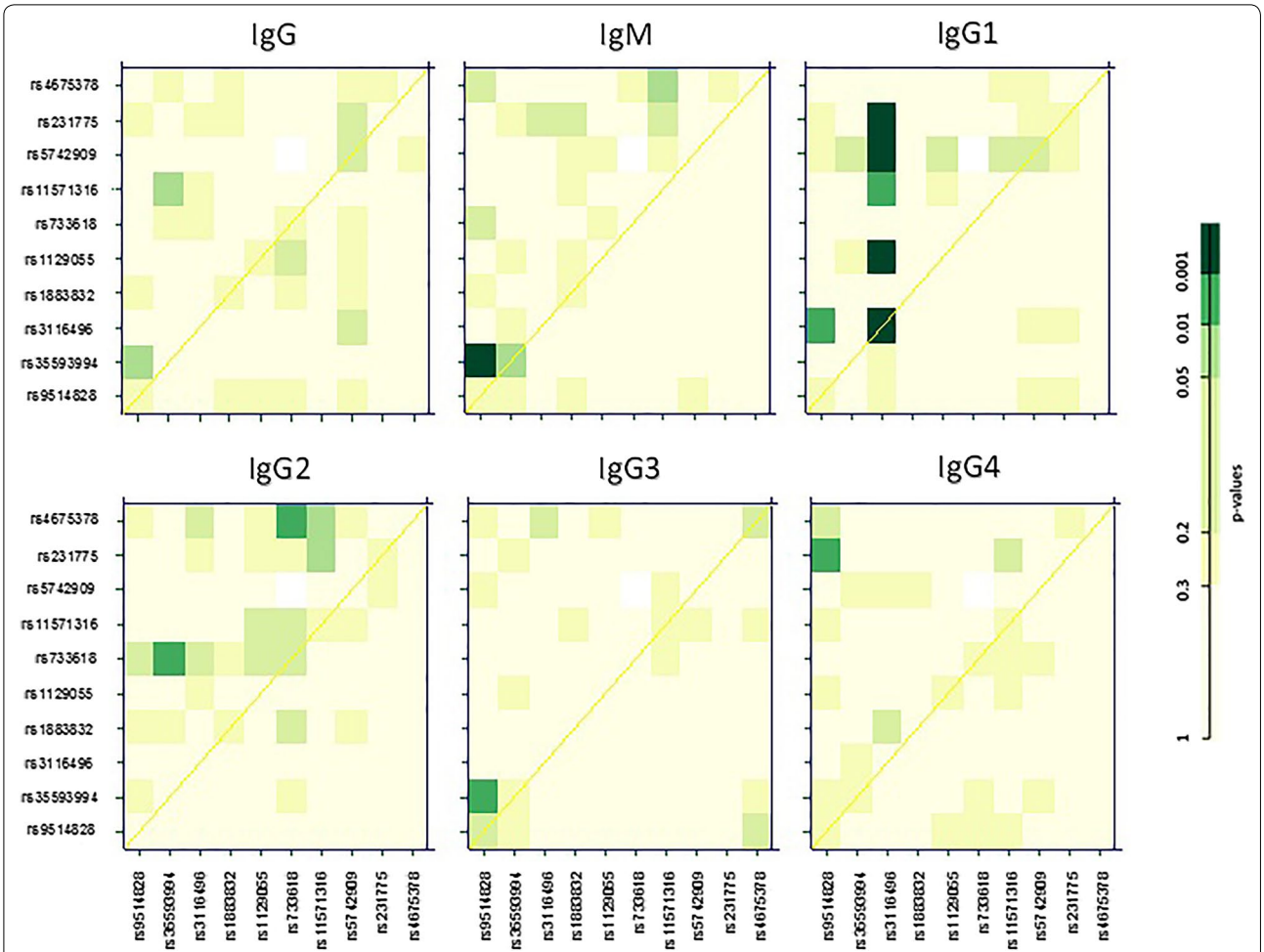

Fig. 2 Interaction between SNPs in the codominant model. The diagonal contains the *p* values of the likelihood ratio test (LRT) for the effect of each SNP individually. The *upper triangle* in the matrix contains the *p* values for the interaction log-LRT. The *lower triangle* contains the *p* values from LRT comparing the two-SNP additive likelihood to the best finding of the single-SNP model [35]

Table 3 Significant interactions between the SNPs rs35593994 and rs9514828 and the levels of IgM specific for ICB2-5

CD28 (rs35593994)	*BLYS* (rs9514828)	IgM (RI[a])	Δ[b]	95 % CI	*p*[c]
GG	CC	0.503	Reference		
GG	CT	1.025	0.523	(0.195–0.850)	0.002
GA	CC	0.916	0.413	(0.126–0.702)	0.005
GA	TT	1.441	0.939	(0.395–1.483)	0.0009

[a] Antibody levels are expressed as the mean of the reactivity index (RI)

[b] Difference of the RI mean

[c] Based on a logistic regression model using individuals exhibiting wild type genotypes (*GG* and *CC*) as a reference

of MSP-1 provide clinical protection during infections with both *P. falciparum* [46, 47] and *P. vivax* [9, 11]. The results of the IgG-mediated humoral immune response showed that ICB2-5 was detected by just over half of the studied individuals (50.6 %). This prevalence of responders is similar to that found in individuals infected with *P. vivax* in other places of the Brazilian Amazon [8, 11]. A small percentage (~10 %) of the individuals who reported having had several previous episodes of malaria exhibited high IgM levels, but no IgG was evidenced, suggesting that class switching from IgM to IgG may be impaired. This observation has been reported in previous studies [8, 48], and Soares et al. [8] suggested that this impaired switch from IgM to IgG may be related to deficient CD40/CD40-L interactions. Thus, the authors evaluated

whether co-stimulatory molecule gene polymorphisms are involved in the delayed class switch from IgM to IgG. However, no association was observed.

Regarding IgG subclasses, higher levels of IgG1 were found compared to the other IgG subclasses, which is in contrast to previous studies showing a predominance of IgG3 specific for ICB2-5 [8, 9, 11]. Although there is still no consensus regarding the role of antibody subclasses in clinical protection, it has been suggested that only the cytophilic subclasses, i.e., IgG1 and IgG3, are protective [49, 50]. Two longitudinal studies performed in the Brazilian Amazon, specifically, one in Portuchuello, near Porto Velho, and the other in a community of Rio Pardo, Amazonas state, have observed that ICB2-5-specific antibodies were associated with clinical protection against malaria caused by *P. vivax* and that IgG3 was detected in all asymptomatic individuals, whereas most symptomatic patients exhibited no IgG3 [9, 11]. If the above results were to be extrapolated to Goianésia do Pará, the site of this study, the fact that a passive collection was performed on patients exhibiting symptoms could explain the low IgG3 levels found. Furthermore, some studies have also shown that IgG2 may be associated with protection. Deloron *et* al. [51] found an association between high IgG2 levels and low risk of acquiring an infection by *P. falciparum*. Although the design of the present study does not allow for the association of the prevalence and levels of clinically protective antibodies, higher IgG2 levels were observed in subjects who reported having had fewer cases of malaria.

The development of an immune response against *Plasmodium* species is a complex process, and one of the main issues is understanding why individuals differ in their immune responses against the parasite. Thus, the objective of the present study was to investigate the influence of co-stimulatory molecule gene polymorphisms on the production of antibodies specific for an important *P. vivax* vaccine candidate antigen. The most important result was that *CD28* rs3116496 was associated with levels of IgG1 specific for ICB2-5. In addition to the important role of CD28 in T cell activation, the binding of this receptor to its ligands CD80 and CD86 on the surface of B cells provides bidirectional signals that appear to be important for IgG production by B cells [52]. Thus, CD28 may be involved in the immune response against malaria. In fact, *CD28* knockout mice infected with *Plasmodium chabaudi* were unable to resolve the infection, maintaining low levels of parasitaemia for weeks after infection [53, 54]. Furthermore, treatment of wild type mice with monoclonal anti-CD86 antibodies abolished IL-4 production and was significantly associated with reduced levels of *P. chabaudi*-specific IgG1 [55].

In the present study, individuals exhibiting the *T* allele for rs3116496 in *CD28* were found to be associated with reduced IgG1 levels. Although the biological functions of this SNP, which is located in the third intron of the gene, are still unknown, it is located near a splice site, at which point mutations can induce abnormal splicing, thus affecting protein expression [56]. The relationship between rs3116496 in *CD28* and susceptibility to several diseases has already been evaluated, and significant associations have been found in type 1 diabetes [57], cervical [58] and breast cancer [59], and rheumatoid arthritis [60]. However, the role of this polymorphism in malaria has not yet been assessed, and even if the presence of the *T* allele, which is associated with lower levels of ICB2-5-specific IgG1, were implicated in higher susceptibility to vivax malaria, further elucidation would still be necessary.

Although it seems likely that immunity against malaria is affected by several genes, the influence of combined polymorphisms is rarely ever investigated. The present study performed analyses of interactions between the SNP pairs and found that *CD28* rs35593994 and *BLYS* rs9514828 together were associated with levels of IgM specific for ICB2-5, especially in individuals with *GA* and *TT* genotypes, respectively. Specifically, they were associated with a ~180 % increase in IgM levels compared to that of wild type genotype individuals. Although the biological mechanisms underlying this interaction are still unknown, CD28 and BLyS may be involved in the production of memory B cells and antibody isotype class switching [61, 62]. Both polymorphisms, i.e., *CD28* rs35593994 and *BLYS* rs9514828, are located in the 5′ regulatory region of the gene; thus, differential expression of these genes may change the regulation of the B cells involved in the production of ICB2-5-specific antibodies. Liu et al. [63] have shown that despite the generation of memory B cells as a response to vaccination with MSP-1_{19}, the function of these cells was nullified due to a lack of BLyS expression in dendritic cells from mice infected with *P. yoelii*.

Although it was observed the influence of these two SNPs in the antibody response to ICB2-5, most of the evaluated polymorphisms did not show any significant differences. In fact, variations in the immune response to malaria can be attributed to several factors, including the environment, previous exposure to malaria and immunogenicity of antigen. Thus, as a complex trait, it is likely that many polymorphisms have small effect in malaria immune response, but large sample size is required to detect it.

Conclusions

In summary, several genes involved in the control of the immune response were investigated, entailing the production of antibodies against a *P. vivax* vaccine candidate antigen. Despite the growing number of studies evaluating human genetic factors that control the immune response to malaria [27–34, 64], it is likely that many genes responsible for the wide inter-individual variation observed in the immune response against malaria remain unknown; studies similar to the present work may help identify subjects who are more prone to respond to a specific vaccine [29]. Although the statistical evidence supporting the described associations is limited by the relatively small sample size and, although it is impossible to tell whether the SNPs studied here are truly causal or are in LD with the causal variants, the results suggest that genetic variations in *CD28* and interactions between polymorphisms in *BLYS* and *CD28* may be involved in the control of the immune response against vivax malaria.

Additional files

Additional file 1. Reaction conditions for the amplification and enzyme digestion of polymorphisms in the studied polymorphisms.

Additional file 2. Correlation between ICB2-5-specific IgG, IgM and IgG subclasses in individuals infected with *Plasmodium vivax* living in an endemic area of the Brazilian Amazon.

Additional file 3. IgM and IgG subclasses levels (RI) for ICB2-5 according to age, length of residence in the studied area and previous episode of malaria.

Additional file 4. Association tests between genetic polymorphisms and *Plasmodium vivax* ICB2-5-specific antibody levels.

Authors' contributions

GCC, RLDM and PAN contributed to the design and conception of the study and wrote the manuscript. AACF, MPC and GCC participated in the molecular analyses. PAN, MEA, DRLB and GCC performed the serum analyses. GCC performed the statistical analyses. LMSM and MMP contributed to data interpretation and reviewed the manuscript. RLDM, GCC and MMP participated in field data collection. All authors read and approved of the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Impact of population admixture on the distribution of immune response co-stimulatory genes polymorphisms in a Brazilian population

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ABSTRACT

Co-stimulatory molecules are essential in the orchestration of immune response and polymorphisms in their genes are associated with various diseases. However, in the case of variable allele frequencies among continental populations, this variation can lead to biases in genetic studies conducted in admixed populations such as those from Brazil. The aim of this study was to evaluate the influence of genomic ancestry on distributions of co-stimulatory genes polymorphisms in an admixed Brazilian population. A total of 273 individuals from the north of Brazil participated in this study. Nine single nucleotide polymorphisms in 7 genes (*CD28*, *CTLA4*, *ICOS*, *CD86*, *CD40*, *CD40L* and *BLYS*) were determined by polymerase chain reaction-restriction fragment length polymorphism. We also investigated 48 insertion/deletion ancestry markers to characterize individual African, European and Amerindian ancestry proportions in the samples. The analysis showed that the main contribution was European (43.9%) but also a significant contribution of African (31.6%) and Amerindian (24.5%) ancestry. *ICOS*, *CD40L* and *CD86* polymorphisms were associated with genomic ancestry. However there were no significant differences in the proportions of ancestry for the other SNPs and haplotypes studied. Our findings reinforce the need to apply AIMs in genetic association studies involving these polymorphisms in the Brazilian population.

1. Introduction

The development of an immune response depends on a complex network of cells and is essential to protect humans against infectious agents and the appearance of autoimmune diseases and tumors. T cells have a critical role in the development of the immune response however these cells require two independent signals for them to become completely activated. The first signal is triggered by the binding of the T cell receptor (TCR) to an antigenic peptide presented by a major histocompatibility complex molecule (MHC). The second signal is provided by co-stimulatory molecules; the binding of the CD28 receptor to CD80 and CD86 molecules is essential for the activation of T cells. However, another molecule called CTLA-4 can also bind to CD80 and CD86 molecules which, instead to providing a positive stimulation, exert a regulatory role by reducing the generated response. This process is crucial for homeostasis and immune tolerance [1].

Another stimulatory receptor expressed on the surface of T cells is called *ICOS*; the gene of this molecule is located close to the *CD28* and *CTLA4* genes in the 2q33 chromosomal region. The interactions between B cells and activated T cells, mediated by CD40/CD40L signaling, also indirectly acts on T cell activation, but this signaling

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pathway is critical to B lymphocyte activation and so, in the development of the humoral immune response. Another molecule, *BLyS*, expressed on the surface of T cells and also in soluble form, modulates the survival and proliferation of B cells through three different receptors: BR3, TACI and BCMA [2].

An adequate immune response must maintain a balance between the ability to respond to infectious agents and to suppress autoimmunity. Thus, polymorphisms associated with the modulation of gene expression of co-stimulatory molecules can influence the development of several diseases. In recent years, several studies have shown associations between polymorphisms in co-stimulatory genes and diseases [3–14]. However, studies in different populations have failed to reproduce the results [15–19]. One of the reasons may be due to variable allele frequencies in different populations, which result in a lack of statistical power. For example, geographical gradients in the distribution of *CTLA4* alleles have been well documented [20]. Population structure also has been presumed to cause many of the unreplicated disease-marker associations reported in the literature, particularly in admixed populations.

Brazil has one of the most diverse populations in the world resulting from five centuries of interethnic breeding between Europeans, Africans and Amerindians. It has been shown that due to the intense miscegenation of the Brazilian population, indicators of physical appearance, such as skin color, are poor indicators of genomic ancestry [21,22]. Some studies have shown that the distribution of pharmacogenetic polymorphisms in the Brazilian population is best characterized using ancestry informative markers (AIMs) instead of self-declaration of ethnicity [23,24]. In fact, nowadays it is recognized that ethnicity can be better studied with AIMs, which enable a better understanding of the relationship between the various ethnic components and the variability of these co-stimulatory genes. Thus, the objective of the present study was to describe the allele frequencies of nine SNPs distributed across seven co-stimulatory genes (*CD28*, *CTLA4*, *ICOS*, *CD86*, *CD40*, *CD40L* and *BLYS*) and assess the impact of Brazilian population admixture on the distribution of these polymorphisms using AIMs.

2. Materials and methods

2.1. Sample

The sample of this study was composed of 273 (175 men and 91 women) unrelated subjects from the town of Goianésia do Pará (03°50′33″ S; 49°05′49″W), located in the southeastern region of the State of Pará in the north of Brazil. All the participants signed informed consent forms. The project was approved by the Research Ethics Committee of the Medicine School in São José do Rio Preto (FAMERP 45992011). The DNA was extracted from peripheral blood samples using the Easy-DNA™ extraction kit (Invitrogen, California, USA).

2.2. Genotyping

The following SNPs were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP): rs35593994 and rs3116496 in the *CD28* gene; rs5742909 and rs231775 in the *CTLA4* gene; rs4404254 in the *ICOS* gene; rs1129055 in the *CD86* gene; rs3092945 in the *CD40L* gene; rs1883832 in the *CD40* gene and; rs9514828 in the *BLYS* gene. All PCR reactions were performed in a final volume of 25 μL containing 1× Buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 pmol of each primer and 0.5 U of Taq DNA Polymerase Platinum (Invitrogen, São Paulo, Brazil). Amplifications were made in a MasterCycler DNA thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: an initial step of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at an annealing temperature depending on the primer and 1 min at 72 °C, and a final step of 10 min at 72 °C. The sequences of the primers as well as the annealing temperatures used in each reaction are shown in Table 1. The amplification products were viewed in agarose gel

Table 1
Location of SNPs, primers, annealing temperatures, restriction enzymes used for genotyping and length of fragments resulting from PCR-RFLP.

SNP	Gene (Chromosome region)	Location	Primer 5′–3′ (forward)	Primer 5′–3′ (reverse)	Annealing temperature (°C)	Restriction enxyme	Fragments length (pb)
rs35593994	*CD28* (2q33)	204570826	TTCTCATTCTGTTGCCCTGGC	CACCATCCCCTTAGGGCACAT	62	HinfI	G: 468 + 78 A: 546
rs3116496	*CD28* (2q33)	204594512	GAAACACCTTTGTCCAAGTC	CTCAATGCCTTCTGGGAAATC	52	AciI	T: 333 C: 193 + 140
rs5742909	*CTLA4* (2q33)	204732347	GGGATTTAGGAGGACCCTTG	GTGCACACACAGAAGGCACT	48	MseI	C: 244 T: 179 + 65
rs231775	*CTLA4* (2q33)	204732714	CTGAACACCGCTCCCATAAA	CACTGCCTTTGACTGCTGAA	50	BbvI	A: 215 G: 159 + 56
rs4404254	*ICOS* (2q33)	204819570	TTACCAAGACTTTAGATGCTTTCTT	GAATCTTTCTAGCCAAATCATATTC	55	AluI	T: 385 + 339 + 99 C: 339 + 289 + 99 + 96
rs1129055	*CD86* (3q21)	121838319	CTGTTCCAATGGCAACCTCT	GGTTGCCCAGGAACTTACAA	56	CviKI-1	G: 79 + 75 + 58 + 54 A: 154 + 58 + 54
rs3092945	*CD40L* (Xq26)	135729609	ATCTTCACAGCAACCTAC	CACTAAACTCAATGAAAGCC	56	LweI	T: 251 + 195 C: 446
rs1883832	*CD40* (20q12-q13.2)	44746982	GAAACTCCTGCGCGGTGAAT	GAAACTCCTGCGCGGTGAAT	56	StyI	C: 133 + 96 + 74 T: 207 + 96
rs9514828	*BLYS* (13q32-q34)	108921373	TGGCTCTTGTGTGATCAAGG	GCCTGGTCTCAGCTTTTCTG	50	MbiI	C: 162 + 48 T: 210

Chromosome positions were referred to the sequence of NCBI database (GRCh37).

Table 2
Genotypic frequencies and proportion of the African, European and Amerindian ancestry according to genotype.

Genotypes	Frequency (%)	African[a]	European[a]	Amerindian[a]
rs35593994	*n = 273*			
G/G	46.7	0.309 (0.29–0.33)	0.445 (0.42–0.47)	0.246 (0.23–0.26)
G/A	45.8	0.320 (0.30–0.34)	0.440 (0.42–0.46)	0.239 (0.22–0.26)
A/A	7.5	0.333 (0.29–0.37)	0.394 (0.35–0.44)	0.273 (0.24–0.31)
p		0.54	0.2	0.33
rs3116496	*n = 273*			
T/T	65.0	0.317 (0.30–0.33)	0.434 (0.42–0.45)	0.248 (0.23–0.26)
T/C	32.0	0.311 (0.29–0.33)	0.453 (0.43–0.48)	0.235 (0.21–0.26)
C/C	3.0	0.349 (0.27–0.42)	0.380 (0.28–0.48)	0.271 (0.18–0.37)
p		0.67	0.2	0.44
rs5742909	*n = 271*			
C/C	0.84	0.317 (0.30–0.33)	0.439 (0.42–0.45)	0.243 (0.23–0.26)
C/T	0.16	0.310 (0.27–0.35)	0.435 (0.39–0.48)	0.255 (0.22–0.29)
p		0.91	0.95	0.93
rs231775	*n = 272*			
A/A	41.0	0.317 (0.30–0.34)	0.432 (0.41–0.45)	0.251 (0.23–0.27)
A/G	49.0	0.313 (0.30–0.33)	0.441 (0.42–0.46)	0.246 (0.23–0.26)
G/G	9.0	0.335 (0.28–0.39)	0.453 (0.40–0.51)	0.212 (0.18–0.23)
p		0.65	0.68	0.17
rs4404254	*n = 269*			
T/T	44.4	0.301 (0.28–0.32)	0.447 (0.42–0.47)	0.252 (0.23–0.27)
T/C	42.3	0.314 (0.29–0.33)	0.445 (0.42–0.47)	0.240 (0.22–0.26)
C/C	13.3	0.371 (0.33–0.41)	0.394 (0.36–0.43)	0.235 (0.20–0.27)
p		**0.003**	0.054	0.53
rs1129055	*n = 272*			
G/G	61.9	0.322 (0.30–0.34)	0.426 (0.40–0.44)	0.251 (0.24–0.27)
G/A	34.0	0.301 (0.29–0.33)	0.459 (0.44–0.48)	0.231 (0.21–0.25)
A/A	4.1	0.278 (0.20–0.35)	0.459 (0.36–0.56)	0.263 (0.20–0.32)
p		0.34	0.09	0.2
rs1883832	*n = 272*			
C/C	73.6	0.319 (0.30–0.33)	0.436 (0.42–0.45)	0.245 (0.23–0.26)
C/T	22.6	0.301 (0.28–0.33)	0.450 (0.42–0.48)	0.241 (0.22–0.27)
T/T	3.8	0.301 (0.22–0.40)	0.434 (0.38–0.50)	0.258 (0.21–0.31)
p		0.81	0.72	0.87
rs9514828	*n = 271*			
C/C	55.3	0.320 (0.30–0.34)	0.432 (0.41–0.45)	0.248 (0.23–0.26)
C/T	37.9	0.317 (0.30–0.34)	0.447 (0.42–0.47)	0.236 (0.22–0.25)
T/T	6.8	0.289 (0.23–0.35)	0.450 (0.38–0.52)	0.261 (0.21–0.31)
p		0.54	0.60	0.47
rs3092945				
Women	*n = 92*			
T/T	78.0	0.300 (0.27–0.33)	0.453 (0.42–0.48)	0.247 (0.23–0.27)
T/C	18.7	0.326 (0.27–0.38)	0.395 (0.34–0.45)	0.279 (0.24–0.32)
C/C	3.3	0.408 (0.07–0.74)	0.424 (0.18–0.67)	0.169 (0.08–0.23)
p		0.22	0.18	0.10
Men	*n = 180*			
T	90.0	0.314 (0.30–0.33)	0.446 (0.43–0.46)	0.240 (0.22–0.26)
C	10.0	0.373 (0.30–0.44)	0.368 (0.30–0.44)	0.259 (0.20–0.32)
p		**0.02**	**0.008**	0.42

Bold value indicates $p < 0.05$.
[a] Ancestry expressed as mean (95% CI).

after staining with 2% GelRed™ (Biotium, Hayward, USA) and were digested using enzymes of the Fermentas company (Vilnius, Lithuania) according to manufacturer's instructions. The enzymes used, as well as the size of the fragments resulting from the digestion of each polymorphism are shown in Table 1. The digestion products were stained with 2.5% GelRed™ (Biotium, Hayward, USA) and viewed in agarose gel with the exception of the rs1883832 and rs5742909 polymorphisms, which were viewed in 12.5% polyacrylamide gel after staining with ethidium bromide.

2.3. Determination of ancestry

Genotyping to determine ancestry was carried out using 48 INDEL-type markers (insertion/deletion) that have been standardized and validated [25]. The markers were selected employing two main criteria: significant differences in allele frequencies between Africans, Europeans and/or Amerindians ($\geqslant$40%) and located on different chromosomes or in distant physical regions when on the same chromosome. Estimation of the parental ancestry of the Brazilian samples was performed considering three parental populations, which was evaluated by Santos et al. [25]: Africans (from Angola, Mozambique, Zaire, Cameroon, and the Ivory Coast), Europeans (mainly Portuguese), and Native Americans (individuals from indigenous tribes of the Brazilian Amazon region). The PCR reactions were carried out on three multiplex systems, each one containing 16 pairs of fluorescent-labeled primers. Electrophoresis was carried out in an automatic sequencer (ABI PRISM 3130 Genetic Analyzer: Applied Biosystems). The sequences of the primers as well as the conditions of cycling and of capillary electrophoresis are described by Santos et al. [25].

2.4. Statistical analysis

The program Structure version 2.3.4 (http://pritch.bsd.uchicago.edu/software.html) was used to estimate the individual interethnic admixture. Allele and genotype frequencies for each variant were obtained using the genetics package [26]. Using this package, deviations from Hardy-Weinberg equilibrium were evaluated by the Chi-square test and the linkage disequilibrium between pairs of loci was analyzed using parameter D'. Haplotype frequencies were estimated by the maximum likelihood method which uses the expectation-maximization algorithm which is part of the haplo.stats package [27]. Analysis of variance (ANOVA) and Student's t test were used to test differences in the proportions of each of the ancestries between different genotypes. A binary logistic regression model was built to graphically explore the association of polymorphisms with individual estimated ancestry using the ggplot2 package [28]. All packages were implemented employing the R computer program, version 2.11.1 (http:www.r-project.org). *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Distribution of polymorphisms according to ancestry

The genotype frequencies of the nine SNPs studied are shown in Table 2. All polymorphisms are in Hardy–Weinberg equilibrium. Allele frequencies found in the current study, as well as in other geographical populations are presented in Supplementary Table 1.

Variance analysis used to test the difference of each ancestry between the different genotypes demonstrated that the mean proportions of African ancestry differed significantly between the genotypes of the rs4404254 SNP in the *ICOS* gene (p-value = 0.003). The Tukey post hoc test indicated that the mean African ancestry was higher for the *CC* genotype than for the *TC* (p-value = 0.01) and *TT* genotypes (p-value = 0.002). As the *CD40L* gene is on the X chromosome, analyses of the rs3092945 SNP in this gene were performed separately for men and women. The mean proportion of African ancestry was higher in men with the *C* allele than those with the *T* allele (p-value = 0.02). Moreover,

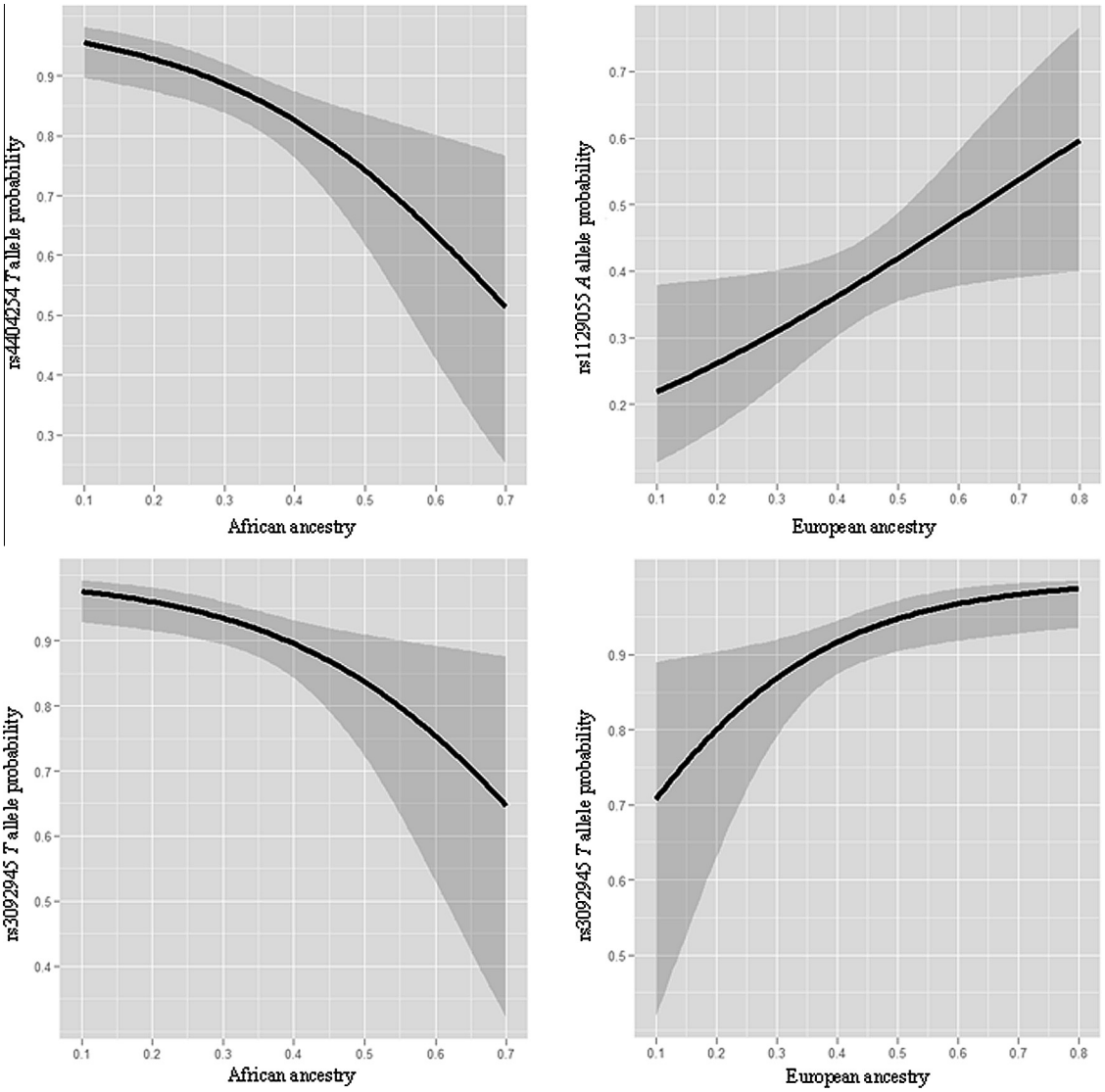

Fig. 1. Fitted logistic regression describing the association between ancestry and polymorphisms. (a) *ICOS* rs4404254. Chance of having a *T* allele according to African ancestry. (b) *CD86* rs1129055. Chance of having a *A* allele according to European ancestry. (c) *CD40L* rs3092945. Chance of having a *T* allele according to African ancestry and (d) according to European ancestry. Gray shadows show 95% confidence intervals. Graphics were created using ggplot2 in R.

men with the *T* allele had a higher average proportion of European ancestry compared to men with the *C* allele (p-value = 0.008). There were no significant differences in the proportions of ancestry for the other SNPs studied (Table 2).

Binary logistic regression, using the generalized linear model and implemented in the program R, was employed to graphically explore the association between polymorphisms and ancestry. The results, presented in Fig. 1, show that the chance of having at least one *T* allele for the rs3092945 of the *CD40L* gene continuously decreases as African ancestry increases (p-value = 0.008). Furthermore, the chance of having this allele increases as the European ancestry increases (p-value = 0.01). In relation to the rs4404254 SNP in the *ICOS* gene, the chance of having the *T* allele decreases as African ancestry increases (p-value = 0.001). The chance of an individual having the *A* allele (rs1129055) in the *CD86* gene increases as the European ancestry increases (p-value = 0.02).

3.2. Linkage disequilibrium and haplotypes

Linkage disequilibrium were evaluated using the statistical parameter D', between all pairs of SNPs in the *CD28, CTLA4* and *ICOS* genes located in the chromosome 2q33 region. There was absolute linkage disequilibrium (D') only between the rs35593994 and rs3116496 SNPs and between the rs35593994 and rs5742909 SNPs. The value of D' varied for the other pairs of SNPs (Supplementary Table 2).

Eighteen haplotypes of the *CD28, CTLA4* and *ICOS* genes were found in the study sample with frequencies ranging from 0.002 to 0.194 (Table 3). The haplo.stats computer program whose function haplo.score generates a score for each haplotype (hap.score), as well as a p-value for each hap.score was used to assess whether a given haplotype is associated with differences in the proportions of ancestry. A positive/negative score for a given haplotype suggest that the haplotype is associated with an increase/decrease in ancestry. Only haplotypes with frequencies higher than 0.01 were included in the analysis. The GTCGT, GTCGC and ATCGC haplotypes had significant associations with African ancestry, and the GTCGT haplotype presented a significant association with European ancestry. However, when the Bonferroni correction was applied (corrected p-value < 0.004), these differences were no longer significant.

Table 3
Haplotype frequencies and association with African, European and Amerindian ancestry.

Haplotype[a]	Frequency[b]	African		European		Amerindian	
		hap. score[c]	p	hap. score[c]	p	hap. score[c]	p
GTCGT	0.193	−2.06	0.03	2.18	0.02	−0.29	0.77
GTCAT	0.153	−1.64	0.09	0.46	0.64	1.37	0.17
ATCAT	0.136	−1.13	0.25	0.17	0.85	1.09	0.27
GTCAC	0.102	1.08	0.27	−0.66	0.50	−0.43	0.66
ATCAC	0.098	1.92	0.05	−1.71	0.08	0.01	0.98
GCTAT	0.055	−0.98	0.32	0.03	0.97	1.11	0.26
GTCGC	0.051	2.26	0.02	−1.07	0.28	−1.41	0.15
GCCAT	0.050	1.15	0.24	−0.55	0.58	−0.69	0.48
GCCAC	0.044	−0.29	0.77	0.44	0.65	−0.24	0.80
ATCGT	0.032	0.07	0.93	0.16	0.87	−0.26	0.78
ATCGC	0.027	2.57	0.01	−1.62	0.10	−0.92	0.35
GCCGT	0.024	0.50	0.61	0.62	0.53	−1.30	0.19
GTTAT	0.008	–	–	–	–	–	–
GCTAC	0.007	–	–	–	–	–	–
ATTAC	0.006	–	–	–	–	–	–
ACTGC	0.002	–	–	–	–	–	–
ATTGC	0.002	–	–	–	–	–	–
GCCGC	0.002	–	–	–	–	–	–

[a] Order of variants in haplotype is as follows rs35593994, rs3116496, rs5742909, rs231775, rs4404254.
[b] Estimated frequency of each haplotype in the population.
[c] The score for the haplotype, which is the statistical measurement of association of each specific haplotype with the trait.

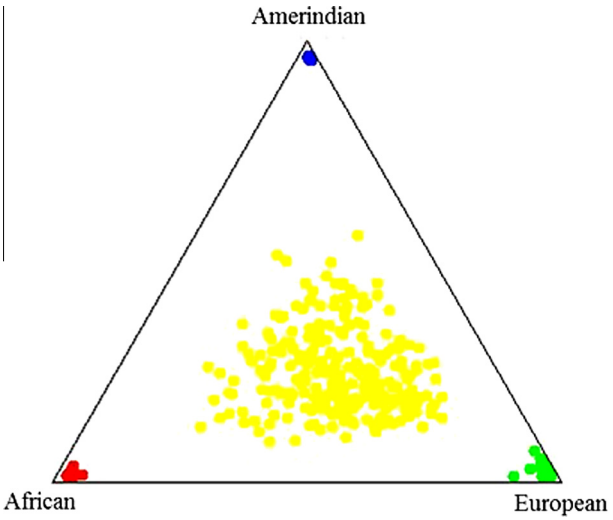

Fig. 2. Schematic representation of the individual admixture estimates. Each point represents one individual and the correspondent admixture proportions are indicated by the distance to the edges of the triangle. European, African and Amerindian correspond to individuals from the parental populations. The figure was made using *Structure* v. 2.3.4 software.

3.3. Estimate of ancestry

The genotypes of the sample population from Goianésia do Pará and parental populations (Europeans, Africans and Amerindians) were analyzed together, assuming $K = 3$. The analysis showed that the study sample is composed of individuals who possess an average of 43.9% European ancestry (ranging from 16.2% to 70.5%), 31.6% African (ranging from 11.4% to 66.4%) and 24.5% Amerindian (ranging from 8.3% to 57.3%). The results are shown in Fig. 2. There were no significant differences in the proportions of genomic ancestry between males and females (Mann–Whitney test, all p-value > 0.14).

4. Discussion

Analysis using AIMs demonstrates that the population of Goianésia do Pará, a town located in the north of Brazil, presents greater contribution from European ancestry (43.9%), and smaller, albeit significant, contributions from African and Amerindian ancestries (31.6% and 24.5%, respectively). These values are similar to other Brazilian populations, although the proportion of African ancestry in this study was higher than other populations of the northern region of Brazil with contributions ranging from 12% to 25% [25,29,30]. This variation might be explained by the large presence of individuals from the northeastern region of Brazil in the studied population, where the contribution of African ancestry is admittedly greater than in other regions of Brazil [21].

We report that *ICOS*, *CD40L*, and *CD86* polymorphisms were associated with genomic ancestry. In fact, according to available data from 1000Genomes project, the *T* allele frequency for rs4404254 SNP in the *ICOS* gene is lower in populations of African origin [31]. The rs3092945 SNP is also significantly associated with ancestry; the *T* allele frequency has a reverse relationship with African ancestry and its frequency increases with the increase in European ancestry. These observations are consistent with available data which show a lower prevalence of this allele in African populations compared to Europeans, specifically Italians and Iberians, who were the largest source of Brazilian immigration [31].

Using a logistic regression model, we showed that the chance of an individual possessing the *A* allele for rs1129055 SNP in the *CD86* gene is enhanced with the increase in European ancestry. This polymorphism has often been assessed in studies of associations with autoimmune diseases and cancer in Asian populations [32–34], but information about the frequency of this SNP in other populations are scarce. Our results are in accordance with data which report a higher frequency of the allele in the European population compared to an African population [31]. Beltrame et al. [35] evaluated this polymorphism in populations of different ancestries and found that the *G* allele is more common, with the exception of the Japanese population, where an inversion of the allele frequencies exists with the *A* allele being the most prevalent. As some Amerindian groups have lower frequencies of the *A* allele, the authors suggest that this change in allele frequencies occurred recently on the Asian continent. Although the frequency of the allele is lower in Amerindian populations, we found no significant association in respect to this allele with Amerindian ancestry.

We found no association of ancestry with the other evaluated SNPs. Previous studies on the Brazilian population compared allele frequencies of polymorphisms between Euro-and Afro-Brazilians (Supplementary Table 1). Differences were only found with the rs3116496 and rs1883832 SNPs [36,37]; this is not in accordance with our results. These differences may be due to the fact that in these studies, the classification of Euro- and Afro-Brazilians was performed using morphological features and/or self-declaration of ethnicity, indicators that have been demonstrated as poor to describe genomic ancestry [21,22]. However, differences in allele frequencies of these two SNPs, as well as the rs9514828 SNP in the gene *BLYS* are evident when the 1000Genomes data are assessed. Hence, it is possible that these populations differ in their allele frequencies compared to the parental populations that formed the population of Goianésia do Pará, which could thus explain the absence of any association of these polymorphisms with ancestry in our study.

The tests of associations between ancestry and haplotypes showed no significant effect of the stratification of the population on the distribution of haplotypes. This corroborates the study of Pincerati et al. [38], who also found no significant differences in the frequencies in the haplotype frequencies of the *CD28* and *CTLA4* genes between Euro- and Afro-Brazilians. Although Butty et al. [39] demonstrated differences in the distribution of haplotypes of the *CD28*, *CTLA4* and *ICOS* genes between different geographical populations, the intense process of miscegenation of the Brazilian population may have eliminated patterns of linkage disequilibrium in parental populations and changed the haplotype frequencies.

Information on genotype and allele frequencies, as well as estimates of haplotype frequencies and their associations with the levels of ancestry are fundamental in mixed populations, since the population structure can lead to spurious results in genetic association studies. In this study we describe the association of SNPs in *ICOS*, *CD40L*, and *CD86* genes with ancestry in the Brazilian population. Our findings reinforce the need to apply AIMs in genetic association studies involving these polymorphisms in the Brazilian population.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humimm.2015.09.045.

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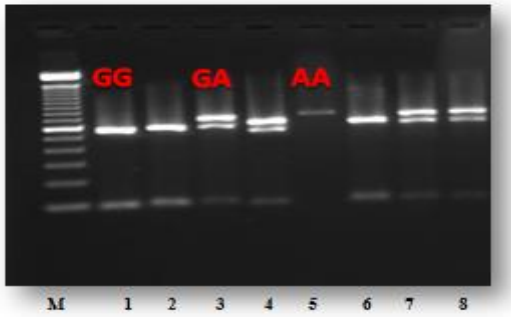
APÊNDICE XVI

Polimorfismos no gene *CD28*

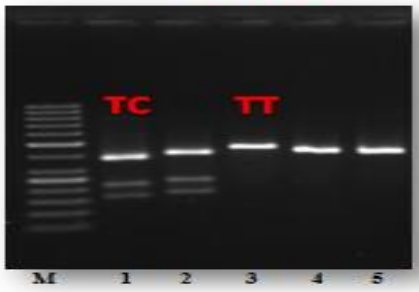
-372G/A



Enzima: HinfI.

M: Ladder de 100 pb
Faixas 1, 2, 6: **GG (468 pb e 78 pb)**
Faixas 3, 4, 7, 8 : **GA (546, 468, 78 pb)**
Faixa 5: **AA (546 pb)**

+17T/C



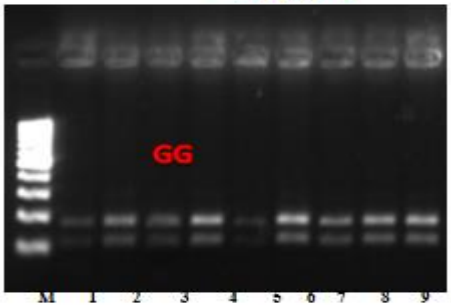
Enzima *AciI*

M: Ladder de 50 pb
Faixas 1, 3, 4 e 5: **TT (333 pb)**
Faixa 2: **TC (333, 193, 140 pb)**

APÊNDICE XVII

Polimorfismos no gene *IFNG*

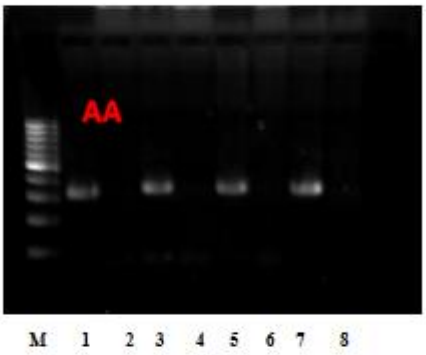
-183G/T



Enzima: Eco47I.

M: Ladder de 100 pb.
Faixas 1 – 9 : **GG (164 pb e 107 pb)**

+874 (T/A)



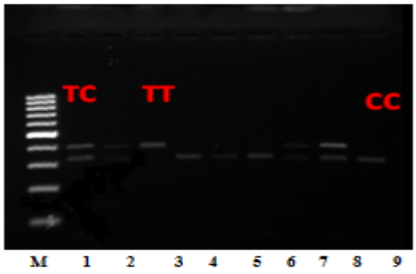
ASO- PCR

M: Ladder de 100 pb.
Faixas 1, 3, 5 e 7: **alelo A (264 pb)**

APÊNDICE XVIII

Polimorfismos no gene da *IL10*

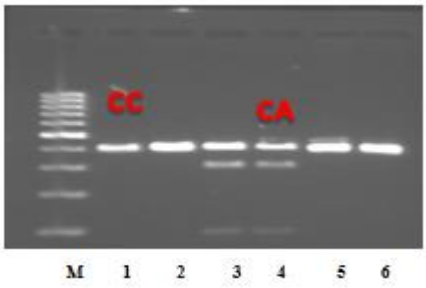
-819 C/T



Enzima: RseI
M: Ladder de 100 pb

Faixas 1, 2, 7 e 8 : **TC (270,217 pb)**
Faixa 3: **TT (270 pb)**
Faixas 4-6 e 9: **CC (217 pb)**

-592 C/A



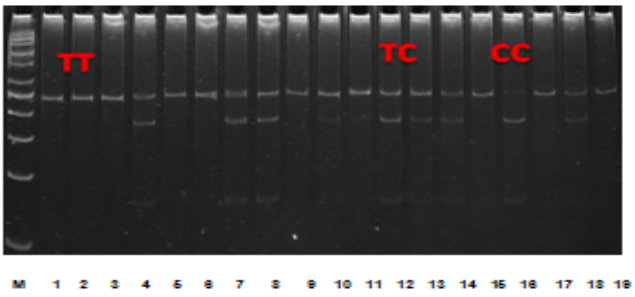
Enzima: RsaI
M: Ladder de 100 pb

Faixas 1, 2, 5 e 6 : **CC (317 pb)**
Faixas 3 e 4 : **CA (317, 240 e 77 pb)**

APÊNDICE XIX

Polimorfismos no gene *TNFA*

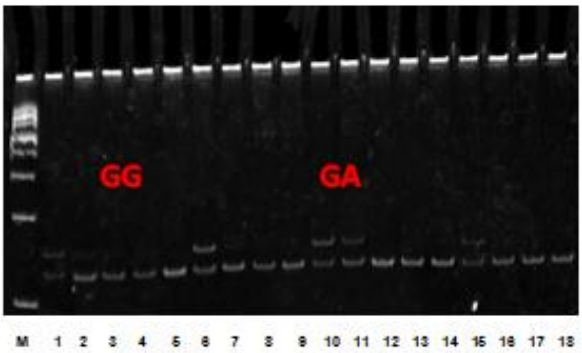
-1031T/C



Enzima: BbsI
M: Ladder de 50 pb.

Faixas 1-3,5,6,9-11,15,17,19: **TT (251 pb)**
Faixas 4, 7,8,10, 12-14, 18: **TC (251 e 180 pb)**
Faixa 16: **CC (180 e 70 pb)**

-308G/A

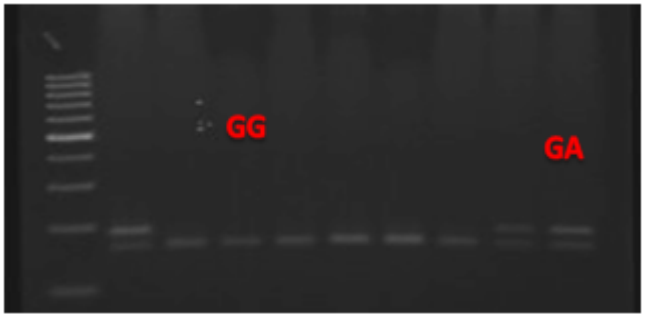


Enzima: NcoI
M: Ladder de 100 pb.

Faixas 1, 6, 10, 11 e 15 : **GA (147 e 126 pb)**
Faixas 2-5, 7-9, 12-14, 16-18: e 4 : **GG (126pb)**

Polimorfismos no gene *TNFA*

-238G/A



M 1 2 3 4 5 6 7 8 9

Enzima: MspI
M: Ladder de 100 pb.

Faixas 2-7: **GG (133 pb)**
Faixa 1, 8 e 9: **GA (153 e 133 pb)**

ANEXOS

ANEXO I

Comitê de Ética em Pesquisa: Utilização de amostras

FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO
Autarquia Estadual - Lei n.° 8899 de 27/09/94
(Reconhecida pelo Decreto Federal n.° 74.179 de 14/06/74)

Parecer n.° 407/2011

COMITÊ DE ÉTICA EM PESQUISA

O Protocolo CEP nº **4599/2011** sob a responsabilidade de **Ricardo Luiz Dantas Machado**, com o título "Influência de polimorfismos em genes associados na produção de anticorpos contra diferentes antígenos candidatos à vacina em indivíduos com Malária Vivax no Estado do Pará" está de acordo com a Resolução do CNS 196/96 e foi **aprovado por esse CEP.**

Lembramos ao senhor(a) pesquisador(a) que, no cumprimento da Resolução 251/97, o Comitê de Ética em Pesquisa em Seres Humanos (CEP) **deverá receber relatórios semestrais sobre o andamento do Estudo**, bem como a qualquer tempo e a critério do pesquisador nos casos de relevância, além do envio dos relatos de eventos adversos, com certeza para conhecimento deste Comitê. **Salientamos ainda, a necessidade de relatório completo ao final do Estudo.**

São José do Rio Preto, 13 de outubro de 2011.

Prof. Dr. Fernando Batigália
Presidente do CEP/FAMERP

Av. Brigadeiro Faria Lima, 5416 - 15090-000 - São José do Rio Preto - SP - Brasil
Tel. (17) 3201-5700 - Fax (17) 3229-1777 - *www.famerp.br*

ANEXO II

Congresso: XVIII International Congress for Tropical Medicine and Malaria/ XLVIII Congresso of the Brazilian Societt of Tropical Medicine.

Formato: poster

XVIII International Congress for Tropical Medicine and Malaria and XLVIII Congress of the Brazilian Society of Tropical Medicine

XXVIII Brazilian Annual Meeting of Applied Research on Chagas Disease, XVI Brazilian Annual Meeting of Applied Research on Leishmaniasis and III Latin American Congress on Travel Medicine

Certificate

This is to certify that Capobianco, M. P., Cassiano, G. C., Furini, A. A. C. Storti-Melo, L. M., Fraga, V. D., Conceição, L. M., Machado, R. L. D. has attended the XVIII International Congress for Tropical Medicine and Malaria and XLVIII Congress of the Brazilian Society of Tropical Medicine, held in Rio de Janeiro from September 23 to 27, 2012, as Poster presentation: Genetic polymorphisms of B-cell co-stimulatory molecules among malaria vivax patients in Brazilian endemic area

Rio de Janeiro, September 27, 2012.

Professor Pierre Ambrosio Thomaz
President of the XVIII

Professor José Rodrigues Coura
President of the XVIII ICTMM

Professor Claudio Tadeu Daniel-Ribeiro
President of the Scientific Committee of the XVIII ICTMM

Professor Carlos Henrique Nery Costa
President of the SBMT

ANEXO III

ASTMH 61 st Annual Meeting – The American Journal of Tropical Medicine and Hygiene (2012)

Resumo em Anais de Congresso

VOLUME 87 NOVEMBER 2012 NUMBER 5 SUPPLEMENT

702

INDIVIDUAL AND EPISTATIC EFFECTS OF GENETIC POLYMORPHISMS OF CD40, CD40L AND BLYS GENES, CO-STIMULATORY MOLECULES ON SUSCEPTIBILITY TO *PLASMODIUM VIVAX* MALARIA

Gustavo C. Cassiano[1], Marcela P. Capobianco[1], Adriana A. Furini[2], Luciane M. Storti-Melo[3], Valeria D. Fraga[2], Luciana M. Conceicao[2], **Ricardo L. Machado[2]**

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Following the candidate gene approach we analyzed the *CD40, CD40L* and *BLYS* genes that participate of B-cell co-stimulation, for association with *Plasmodium vivax* malaria, characterized by a non-lethal disease but its prolonged and recurrent infection with deleterious effects on personal well-being, growth and on the economic performance at individual, family, community, and national levels. *P. vivax* is the most prevalent malaria species in Brazil it represents more than 80% of clinical cases reported annually from the Amazon region. The parasite-host coevolutionary process can be viewed as an arms race, in which adaptive genetic changes in one are eventually matched by alterations in the other, in this case, within the genetically diverse Amazonian populations. The sample included 97 patients and 103 controls. We extracted the DNA by using the extraction and purification commercial kit and identified the SNPs –1 C>T in the gene *CD40*, -726T>C in the gene *CD40L* and the -871C>T in the gene *BLyS* by the PCR-RFLP method. We analysed the genotypic, allelic frequencies, as well as of those individuals carrying each allele, by direct counting. We also compared the observed genotypic frequencies with the expected ones, according to the Hardy-Weinberg Equilibrium. The allelic, genotypic and allele carrier frequencies for these SNPs did not differ statistically between the patient and the control groups. Gene-gene interactions were no observed between CD40 and BLYS, and between CD40L and BLYS. Overall, the genes were balanced according to Hardy-Weinberg Equilibrium. The results of this study lead us to conclude that, although the CD40, CD40L and BLYS alleles analysed differ functionally, this variation does not alter the functionality of the molecules in a way that would interfere with the susceptibility of the disease.

ANEXO IV

American Society of Tropical Medicine and Hygiene 62st Meeting (2013)

Resumo em Anais de Congresso

671

POLYMORPHISMS IN CO-STIMULATORY GENES DO NOT AFFECT *PLASMODIUM VIVAX* PARASITE DENSITY

Gustavo C. Cassiano[1], Marcela P. Capobianco[1], Adriana A. Furini[2], Luciane M. Storti-Melo[3], Valéria D. Fraga[2], Luciana M. Conceição[2], Pamella C. Trindade[2], Franciele M. Tomaz[2], Ricardo L. Machado[4]

[1]*Universidade Estadual Paulista, São José do Rio Preto, Brazil,* [2]*Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, Brazil,* [3]*Universidade Federal do Sergipe, Itabaiana, Brazil,* [4]*Instituto Evandro Chagas, Belém, Brazil*

Plasmodium vivax is the most prevalent malaria species in Brazil, representing more than 80% of clinical cases reported annually from the Amazon region. A growing body of evidence indicates that the immunity is important in the outcome of *P. vivax* infection. Co-stimulation is an important secondary signal that governs the extent, strength and direction of the immune response that follows. Since parasite density has been recognized as important factor in the outcome in malaria infections, we investigated whether polymorphisms in co-stimulatory genes are associated with *P. vivax parasitemia* in malaria patients from Brazilian Amazon Region. The sample included 147 patients infected with *P. vivax* from Goianésia do Pará, a municipality situated on the southwest of Pará state, Brazil. Nine SNPs were analyzed by PCR-RFLP in seven co-stimulatory genes (BAFF, CD28, CTLA4, CD40, CD40L, CD86 and ICOS). Parasitemia was determined by counting the number of parasites in 100 separate fields under oil immersion microscope and converted to the number of parasites per microliter of blood assuming 8,000 leukocytes/μL. Association between the genotypes and parasite density was determined by Mann-Whitney test, with level of significance of 0.05, using R statistical software. All SNPs tested were in Hardy-Weinberg equilibrium. A trend was noted between the allele C of SNP rs_3116496 in CD28 gene and lower parasite density, but these trend was not significant ($p = 0.1$). No significant association was found between the polymorphisms tested and *P. vivax* parasite density. Our results show that the studied polymorphisms do not affect the *P. vivax* parasite density. However, due to the obvious importance of co-stimulatory pathways in malaria, further studies that elucidate the complex host-parasite interactions could be useful for future vaccine development.

ANEXO V

XIII Reunião Nacional de Malária em Manaus (2013)

Resumo em Reunião

IMU - 08

AMERINDIAN ANCESTRY IN A BRAZILIAN POPULATION IS POSITIVELY CORRELATED WITH ANTIBODY LEVELS TO *PLASMODIUM VIVAX* MEROZOITE SURFACE PROTEIN-1_{19} KDA

Gustavo CCassiano[1,3], Eduardo JM Santos[2], Maria HT Maia[2], Adriana C Furini[3], Franciele MB Tomaz[3], Pamella CA Trindade[3], Marcela P Capobianco[1], Andréa LS Silva[2], Ana Paula G Castro[2], Giselle MR Viana[4], Marinete M Póvoa[4], Maristela G Cunha[2], Sidney EB Santos[2], Ricardo LDMachado[1,4]

1.Universidade Estadual Paulista (Unesp); 2.Universidade Federal do Pará (UFPA); 3.Faculdade de Medicina de São José do Rio Preto (FAMERP);4.Instituto Evandro Chagas (IEC)

Introduction:A growing body of evidence indicates that the immunity is important in the outcome of malaria infection and one of the main goals of immunologists is to understand why people differ in their immune response to the parasite. Although some progress hasbeen made in identifying human genetic factors that influence *Plasmodiumfalciparum* antibody response in African populations, little is known about the factors that participate in humoral immune response to malaria in admixed populations. The population of Brazil is genetically highly heterogeneous, admixed between Europeans,Amerindians, and Sub-Saharan Africans, as a result of the relatively recent history of encounter.**Objective:** In the current study, the effect of Amerindian, European and African ancestry in antibody levels to *P. vivax* MSP-1_{19} was examined in an admixed population in Brazil. **Material and Methods:**Samples were collected of 195 *P. vivax*-infected individuals from a Brazilian malaria endemic area (Goianésia do Pará). IgG antibodies to *P. vivax* MSP-1_{19} were detected by ELISA and 47 INDELs ancestry markers were genotyped to estimate individual ancestry proportions using STRUCTURE software. Spearman correlation was used to assess correlation between ethnic ancestry and antibody levels. **Results:**The Amerindian ancestry correlated positively with antibody levels (rho=0,151, p=0,03). The African and European ancestry were not correlated with antibody levels (p=0,36 and 0,40, respectively). **Conclusion:**Our results indicate that Amerindian populations can be more exposed to *P. vivax* in the Brazilian Amazon.

Funding: CNPq

ANEXO V

Congresso Brasileiro de Medicina Tropical (2013)

Resumo em Anais de Congresso

ÁREA TEMÁTICA: F) DOENÇAS POR PROTOZOÁRIOS

P-713

TÍTULO: MALÁRIA VIVAX EM GOIANÉSIA DO PARÁ, ESTADO DO PARÁ: POLIMORFISMOS DO GENE DA IL4 VERSUS PARASITEMIA

AUTOR(ES): FRANCIELE MAIRA MOREIRA BATISTA TOMAZ , FRANCIELE MAIRA MOREIRA BATISTA TOMAZ, ADRIANA ANTÔNIA DA CRUZ FURINI, GUSTAVO CAPATTI CASSIANO, MARCELA PETROLINI CAPOBIANCO, MARINETE MARINS PÓVOA, PAMELLA CRISTINA ALVES TRINDADE, VALERIA DALTIBARI FRAGA, LUCIANA MORAN CONCEIÇÃO, RICARDO LUIZ DANTAS MACHADO

INSTITUIÇÃO: FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO

MALÁRIA VIVAX EM GOIANÉSIA DO PARÁ, ESTADO DO PARÁ: POLIMORFISMOS DO GENE DA *IL4* VERSUS PARASITEMIA

FRANCIELE MAIRA MOREIRA BATISTA TOMAZ[1], ADRIANA ANTÔNIA DA CRUZ FURINI[1], GUSTAVO CAPATTI CASSIANO[1,2], MARCELA PETROLINI CAPOBIANCO[1,2], MARINETE MARINS PÓVOA[3], PAMELLA CRISTINA ALVES TRINDADE[1], VALERIA DALTIBARI FRAGA[1], LUCIANA MORAN CONCEIÇÃO[1], RICARDO LUIZ DANTAS MACHADO[1, 2, 3].

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Introdução e objetivo: A proteína expressa pelo gene *IL4* ativa as células B, com diminuição da resposta tipo Th1, estimula o crescimento e a diferenciação de eosinófilos. Investigamos as frequências genotípicas deste gene em 85 indivíduos para o VNTR e 35 indivíduos para o SNP -590 C>T com malária vivax residentes no município de Goianésia do Pará e sua correlação com a parasitemia. **Material e Métodos:** a extração de DNA foi utilizada empregando-se o kit de extração/purificação Easy-DNA™ e a identificação do SNP -590 C>T no gene da *IL4* foi efetuada por meio do método de PCR-RFLP e o VNTR por PCR. A parasitemia foi determinada por contagem do número de parasitas em 100 campos separados sob microscópio de imersão em óleo e convertido no número de parasitas por microlitro de sangue assumindo 8.000 leucócitos / microlitro. A associação entre os genótipos e densidade parasitária foi determinada pelo teste de Mann-Whitney, com nível de significância de 0,05, utilizando o software estatístico R. **Resultados:** A frequência maior foi verificada para o genótipo CT (54,3%), seguido do genótipo CC (28,6%) e do genótipo TT (17,1%). Para o VNTR o genótipo as frequências observadas foram B1/B2 (50%), B2/B2 (41,7%), enquanto que o genótipo B1/B1 foi 8,3%. Não houve diferença estatística significante entre as frequências genotípicas do SNP -590 C>T e a repetição em *TANDEM* VNTR. Entretanto, os indivíduos com genótipo TT (-590) apresentaram maior parasitemia (p=0,06). **Conclusão:** Os polimorfismos no gene *IL4* podem contribuir para níveis alterados da citocina, nos níveis de anticorpos e consequentemente na carga parasitária e, portanto, influenciar na susceptibilidade a malária vivax na população do município investigado.

Fonte Financiadora: FAMERP e CNPq

ANEXO VI

Congresso Brasileiro de Medicina Tropical (2013)

Resumo em Anais de Congresso

ÁREA TEMÁTICA: F) DOENÇAS POR PROTOZOÁRIOS

P-753

TÍTULO: POLIMORFISMOS EM GENES CO-ESTIMULATÓRIOS NÃO INFLUENCIAM A RESPOSTA DE ANTICORPOS CONTRA A MSP-119 DO PLASMODIUM VIVAX EM INDIVÍDUOS NATURALMENTE INFECTADOS EM UMA ÁREA ENDÊMICA DA AMAZÔNIA BRASILEIRA

AUTOR(ES): GUSTAVO CAPATTI CASSIANO , GUSTAVO CAPATTI CASSIANO, MARCELA PETROLINI CAPOBIANCO, ADRIANA ANTÔNIA DA CRUZ FURINI, LUCIANE MORENO STORTI-MELO, PAMELLA CRISTINA ALVES TRINDADE, FRANCIELE MAIRA BATISTA TOMAZ, MARISTELA GOMES DA CUNHA, MARINETE MARINS PÓVOA, RICARDO LUIZ DANTAS MACHADO

INSTITUIÇÃO: UNIVERSIDADE ESTADUAL PAULISTA

Introdução: Inúmeras evidências indicam que a imunidade é importante no resultado da infecção por *Plasmodium* e um dos principais objetivos dos imunologistas é entender os motivos pelos quais as pessoas diferem em suas respostas imunes contra o parasito. Assim, é importante elucidar o componente genético envolvido na resposta imune naturalmente adquirida contra a malária. O objetivo do presente trabalho foi avaliar a influência de polimorfismos em genes co-estimulatórios na prevalência e magnitude de anticorpos contra a MSP-1_{19} do *Plasmodium vivax*, em indivíduos naturalmente infectados em uma área endêmica da Amazônia brasileira. **Material e Métodos**: A amostra foi constituída por 189 indivíduos infectados por *P. vivax* provenientes do município de Goianésia do Pará, localizado no sudeste do Estado do Pará, Brasil. Nove SNPs foram analisados por PCR-RFLP em sete genes co-estimulatórios (*BAFF*, *CD28*, *CTLA4*, *CD40*, *CD40L*, *CD86* e *ICOS*). As amostras de plasma foram avaliadas em duplicata por teste de ELISA para presença de anticorpos contra a MSP-1_{19} do *P. vivax* e os resultados foram expressos pelo índice de reatividade (média das densidades ópticas da amostra/ ponto de corte). As análises estatísticas foram realizadas utilizando o software R. Diferenças nas médias foram avaliadas por análise de variância (ANOVA) e Qui Quadrado. Valores de p menores do que 0,05 foram considerados significativos. **Resultados**: Todos os SNPs avaliados estão em equilíbrio de Hardy-Weinberg. A frequência de respondedores contra a MSP-1_{19} do *P. vivax* foi de 82,5%. Os indivíduos com o genótipo TT para o SNP rs1883832 no gene *CD40* ou aqueles apresentando no mínimo um alelo A no SNP rs1129055 no gene *CD86* apresentaram maiores índices de reatividade de anticorpos, sem associação estatisticamente significativa ($p = 0,06$ e $p= 0,08$, respectivamente). Não houve associação significativa entre os genótipos estudados e a frequência de respondedores. **Conclusão**: Apesar dos polimorfismos estudados já terem sido implicados como importantes na resistência ou susceptibilidade a diversas doenças auto-imunes, nosso estudo não evidenciou uma importante função dos mesmos na aquisição ou níveis de anticorpos contra a MSP-1_{19} do *P. vivax* na população estudada.

Apoio Financeiro: CNPq

ANEXO VII

63 rd Annual Meeting – The American Society of Tropical Medicine and Hygiene (2014)

Resumo em Anais de Congresso

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POLYMORPHISM ANALYSIS OF THE CTLA-4 GENE IN *PLASMODIUM VIVAX* MALARIA PATIENTS FROM BRAZILIAN AMAZON REGION

Pamella C. Trindade[1], Gustavo C. Cassiano[2], Franciele M. Tomaz[1], Adriana A. Furini[1], Marcela P. Capobianco[1], Marinete M. Póvoa[3], Valéria D. Fraga[1], Luciana M. Conceição[1], Ricardo L. Machado[1]

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Plasmodium vivax has been the most common cause of the human malaria parasite in the brazilian amazon region. Cell-mediated immunity requires costimulatory activity to initiate or inhibit antigen-specific t-cell responses. CTLA-4 is an inhibitory receptor expressed by activated and regulatory t cells. The aim of this study was to analyze two snps located on promotor region of CTLA4 gene in *P. vivax* patients and correlated it with parasitaemia and IL-4 levels. A total of 188 *P. vivax* malaria patients were enrolled in the study. Dna was extracted from blood samples using dna-easy kit (invitrogen). A PCR-RFLP protocol was used to analyze the genotype and allele frequencies of these polymorphisms. The density of parasitemia in the infected individuals was recorded and expressed as the number of asexual *P. vivax* per microliter of blood assuming a count of 100 microscopy fields and estimated before treatment. The serum levels of IL-4 were detected by milliplex map kit using magpix/luminex®. Analyses were performed using r version 2.8.1 statistical software. For the polymorphism at position -1577 g>a, the g/a genotype had the highest frequency (49.4%), followed by the g/g genotype (41%) and the a/a genotype (9.6%) for the polymorphism at position -1722 t>c, the t/t genotype had the highest frequency (86.7%), followed by the T/C genotype (12.3%) with the least frequent being the C/C genotype (1%). The IL-4 plasma level ranged from 0,61 to 9,32 pg/ml. There were no statistically significant differences either in parasitaemia and plasma il4 levels among individuals with different genotypes. This study showed that there was no association between the CTLA-4 snps with the development of malaria vivax, serum cytokine and peripheral *P. vivax* parasitaemia in brazilian amazon region. The CTLA -4 snps may be associated with malaria vivax in other endemic

ANEXO VIII

63 rd Annual Meeting – The American Society of Tropical Medicine and Hygiene (2014)

Resumo em Anais de Congresso

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IL4 GENE POLYMORPHISMS ARE NOT ASSOCIATED WITH *PLASMODIUM VIVAX* MALARIA IN BRAZIL

Franciele M. Tomaz[1], Gustavo C. Cassiano[2], Adriana A. Furini[1], Marcela P. Capobiano[2], Pamella C. Trindade[1], Marinete M. Póvoa[3], Valéria D. Fraga[1], Lucas R. Azevedo[1], Sônia M. Oliani[2], Luciana M. Conceição[1], Ricardo L. Machado[1]

[1]*Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, Brazil,* [2]*Universidade Estadual Paulista, São José do Rio Preto, Brazil,* [3]*Instituto Evandro Chagas, Belém, Pará, Brazil*

Interleukin 4 (IL-4) is an anti-inflammatory cytokine, which regulates balance between Th1 and Th2 immune response, immunoglobulin class switching and humoral immunity. The present study investigated the influence of polymorphisms in IL-4 gene related to the immune system in patients with malaria caused by *Plasmodium vivax* in Brazilian endemic area. A total of 83 individuals infected by *P. vivax* were genotyped by pcr/rflp for two (-590 c/t, -33 c/t) single nucleotide polymorphisms (snps) and the intron 3 vntr polymorphism pcr method in IL4 gene. The density of parasitemia in the infected individuals was recorded and expressed as the number of asexual *P. vivax* per microliter of blood assuming a count of 100 fields per slide. The serum levels of IL-4 were detected by milliplex map kit (human cytokine/chemokine magnetic bead panel-hcytomag-60k) using magpix/luminex®. Analyses were performed using r version 2.8.1 statistical software. For the polymorphism at position - 590 in the il4 gene, the c/t genotype had the highest frequency (55.4%). For the polymorphism at position -33, the c/t genotype had the highest frequency (51.8%) for the polymorphism at vntr the b1b2 genotype had the highest frequency (50.6%). The genotype frequencies were according to the Hardy-Weinberg equilibrium. The il-4 serum level ranged from 0,61 to 9,32 pg/ml. The parasitemia on the thick blood films ranged from 5 to 15.000 parasites/mm3. There were no statistically significant differences either in parasitaemia, serum il-4 level among individuals with different genotypes and haplotypes. Our findings suggest that il4 gene polymorphisms were not associated with serum cytokine and peripheral *P. vivax* parasitaemia in

ANEXO IX

XXXIX Congress of the Brazilian Society of Immunology

Formato: poster

IMMUNO BÚZIOS 2014

XXXIX Congress of the Brazilian Society of Immunology

VII ESCI - Extra Section of Clinical Immunology

October 18 - 22, 2014
Atlântico Búzios Hotel
Búzios - RJ, Brazil

Organization
SBI Sociedade Brasileira de Imunologia

Certificate

We hereby certify that the scientific papers entitled

ANALYSIS OF IL4 GENE POLYMORPHISMS AND IGG ANTIBODY RESPONSE TO PLASMODIUM VIVAX VACCINE CANDIDATE ANTIGEN PVAMA-1

with the authors

FRANCIELE MAIRA TOMAZ; ADRIANA ANTÔNIA DA CRUZ FURINI; MARCELA PETROLINI CAPOBIANCO; MARINETE MARINS POVOA; PAMELLA CRISTINA ALVES TRINDADE; VALÉRIA DALTIBARI FRAGA; LUCIANA MORAN CONCEIÇÃO; LUCAS RIBEIRO AZEVEDO; SÔNIA MARIA OLIANI; GUSTAVO CAPATTI CASSIANO; CARLOS EUGÊNIO CAVASINI; SIDNEY EMANUEL BATISTA DOS SANTOS; RICARDO LUIZ DANTAS MACHADO.

was presented as **POSTER**
in the **XXXIX Congress of the Brazilian Society of Immunology 2014**

Búzios - RJ, Brazil, October 22nd, 2014.

João P. B. Viola
President

ANEXO X

Med Trop – 51º Congresso da Sociedade Brasileira de Medicina Tropical

Formato: pôster

MedTrop 2015
51º Congresso da Sociedade Brasileira de Medicina Tropical

14 a 17 de junho de 2015
Centro de Eventos do Ceará
Fortaleza/CE - Brasil

Tema Central:
Doenças Tropicais: do ensino e pesquisa aos serviços de saúde

CERTIFICADO

Certificamos que o trabalho

POLIMORFISMOS NO GENE DO RECEPTOR TCR E OS NÍVEIS DE PARASITEMIA NA MALÁRIA VIVAX NA AMAZÔNIA BRASILEIRA

Tendo como autores: Marcela Petrolini Capobianco, Gustavo Capatti Cassiano, Adriana Antônia da Cruz Furini, Valéria Daltibari Fraga, Luciana Moran Conceição, Cláudia Regina Bonini-Domingos, Ricardo Luiz Dantas Machado,
foi apresentado no 51º Congresso da Sociedade Brasileira de Medicina Tropical - MEDTROP,
como Pôster na categoria Doenças por protozoários.

Fortaleza, 29 de maio de 2015.

Mitermayer Galvão dos Reis
Presidente da SBMT

Ivo Castelo Branco Coelho
Presidente do Congresso

Luciano Pamplona de Góes Cavalcanti
Presidente da Comissão Científica

Para verificar a autenticidade deste certificado, acesse o link http://medtrop2015.gnius.com.br/certificado e informe o código: 07026754

ANEXO XI

XIV Reunião Nacional de pesquisa em Malária

Formato: pôster

XIV REUNIÃO NACIONAL DE PESQUISA EM MALÁRIA

30 DE SETEMBRO A 1 DE OUTUBRO DE 2015

CERTIFICADO

Certificamos que o Trabalho **nº PT.069** com o título: "**Polimorfismo no gene da IL2 e os níveis de parasitemia na Malária vivax**", de autoria de *Capobianco M, Cassiano GC, Furini AAC, Fraga VD, Conceição LM, Bonini-Domingos CR, Machado RLD* foi apresentado como **pôster** na XIV Reunião Nacional de Pesquisa em Malária realizada de 30 de Setembro a 3 de Outubro de 2015 no Centro de Convenções Rebouças, São Paulo – SP.

FÁBIO TRINDADE MARANHÃO COSTA (UNICAMP)
PRESIDENTE DA COMISSÃO CIENTÍFICA

MARCELO URBANO FERREIRA (USP)
PRESIDENTE DA COMISSÃO ORGANIZADORA

ANEXO XII

XIV Reunião Nacional de pesquisa em Malária

Formato: pôster

XIV REUNIÃO NACIONAL DE PESQUISA EM MALÁRIA

30 DE SETEMBRO A 1 DE OUTUBRO DE 2015

CERTIFICADO

Certificamos que o Trabalho **CO.01** com o título: "**Polimorfismos em genes coestimulatórios de células B estão associados a resposta de anticorpos IgG contra proteínas de estágio sanguíneo do Plasmodium vivax**", de autoria de *Cassiano GC, Furini AAC, Capobianco MP, Cunha MG, Kano FS, Carvalho LH, Soares IS, Santos SE, Póvoa MM, Machado RLD* foi apresentado como **comunicação oral** na XIV Reunião Nacional de Pesquisa em Malária realizada de 30 de Setembro a 3 de Outubro de 2015 no Centro de Convenções Rebouças, São Paulo – SP.

FÁBIO TRINDADE MARANHÃO COSTA (UNICAMP)
PRESIDENTE DA COMISSÃO CIENTÍFICA

MARCELO URBANO FERREIRA (USP)
PRESIDENTE DA COMISSÃO ORGANIZADORA

ANEXO XIII

Participação em artigos publicados durante o doutorado

1-Galisteu KJ, Cardoso LV, Furini AAC, Schiesari Júnior A, Cesarino CB, Franco C, Baptista ARS, Machado RLD. Opportunistic infections among individuals with HIV-1/AIDS in the highly active antiretroviral therapy era at a Quaternary Level Care Teaching Hospital. Rev Soc Bras Med Trop 2015; 48:149-56.

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6. Verona JP, Verona JAOB, Furini AAC. Gerenciamento de Resíduos de Serviços de Saúde em Indústria de Produtos Hospitalares. Revista Eletrônica de Pesquisa da UNIRP – Universitas 2013; 3: 1-10.

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9. Atique TSC , Lima TAM, Souza VA, Pacheco PFS, Furini AAC. Sensibilidade à meticilina/oxacilina de *Staphylococcus aureus* isolados da mucosa nasal de alunos do Centro Universitário de Rio Preto.. Revista Brasileira de Farmacia 2012; 93: 347-52.

10. Reis AGR, Guimarães PM, Atique TSC, Furini AAC. Análise de potenciais interações medicamentosas em prescrições de um Hospital Veterinário do noroeste paulista como ferramenta do serviço de farmácia hospitalar para reduzir suas reais manifestações. Revista de Ciências Farmacêuticas Básica e Aplicada 2012; 33: 291-98.

11. Atique TSC, Atique Netto HÁ, Morelli JJ, Momesso CAS, Furini AAC. Isolamento de agentes da Mastite Bovina, perfil de sensibilidade e resíduos de

antimicrobianos em São José do Rio Preto - São Paulo, Mastite Bovina e resíduos de antimicrobianos. Revista Eletrônica de Pesquisa da UNIRP – Universitas 2012; 2:29-8.

12. Atique TSC, Furini AAC, Reis AG, Guimarães PM, Atique Netto HA. Indicadores de prescrição e interações medicamentosas no Hospital Veterinário Dr. Halim Atique-UNIRP. Revista de Ciências Farmacêuticas Básica e Aplicada 2012; 33: 291-96.

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