PAMELLA CRISTINA ALVES TRINDADE

POLIMORFISMOS NO GENE *CTLA4* ASSOCIADO COM REGULAÇÃO DE CÉLULAS T EM INDIVÍDUOS NATURALMENTE INFECTADOS PELO *PLASMODIUM VIVAX* NO ESTADO DO PARÁ

São José do Rio Preto
2014

Pamella Cristina Alves Trindade

Polimorfismos no gene *CTLA4* associado com regulação de células T em indivíduos naturalmente infectados pelo P*lasmodium vivax* no Estado do Pará

Dissertação apresentada à Faculdade de Medicina de São José do Rio Preto para obtenção do Título de Mestre no Curso de Pós-Graduação em Ciências da Saúde,
Eixo Temático: Medicina e Ciências Correlatas

Orientador: Prof. Dr. Ricardo Luiz Dantas Machado

São José do Rio Preto

2014

Trindade, Pamella Cristina Alves
Polimorfismos do gene *CTLA4* associado na regulação de células T na resposta imune em indivíduos naturalmente infectados pelo *Plasmodium vivax* no Estado do Pará
São José do Rio Preto, 2014
p. 88

Dissertação (Mestrado) – Faculdade de Medicina de São José do Rio Preto – FAMERP
Eixo Temático: Medicina e Ciências Correlatas

Orientador: Profº Drº Ricardo Luiz Dantas Machado

1. Malária; 2. *Plasmodium vivax*; 3.polimorfismos de base única; 4. *CTLA4*

Pamella Cristina Alves Trindade

Polimorfismos no gene *CTLA4* associado com regulação de células T em indivíduos naturalmente infectados pelo P*lasmodium vivax* no Estado do Pará

BANCA EXAMINADORA
DISSERTAÇÃO PARA OBTENÇÃO DO GRAU DE MESTRE

Presidente e Orientador: Ricardo Luiz Dantas Machado
2º Examinador: Eny Maria Goloni Bertollo
3º Examinador: Luciane Moreno Storti de Melo
Suplentes: Érika Cristina Pavarino Bertelli
Andréa Regina Baptista Rossit

São José do Rio Preto, 04/09/2014.

SUMÁRIO

AGRADECIMENTOS

- Primeiramente a Deus, que fez me superar as dificuldades encontradas no caminho.

- Agradeço aos meus pais, ao meu irmão, meus familiares e todas as pessoas que foram incentivadoras neste processo para a concretização deste estudo.

- Ao Professor Ricardo Luiz Dantas Machado pela oportunidade de trabalhar sob sua orientação, pela sua dedicação, paciência e que mesmo com a distância esteve presente em todas as etapas.

- À coordenação e aos funcionários do Curso de Pós-Graduação em Ciências da Saúde da Faculdade de Medicina de São José do Rio Preto, que sempre estiveram dispostos a melhor atender.

- Aos professores mestres e doutores que repassaram seus conhecimentos, durante o curso.

- Ao professor Carlos Eugênio Cavasini, que nos aceitou e acolheu para darmos continuidade ao trabalho no Centro de Investigação de Microrganismos.

- Às técnicas de laboratório: Valéria Daltibari Fraga e Luciana Moran Conceição pelos ensinamentos e auxílios técnicos/laboratoriais para realização desse trabalho.

- Aos colegas de laboratório: Gustavo Capatti, Adriana Furini, Franciele Maira e Marcela Capobianco, pelo apoio, pelas experiências trocadas, por todos os ensinamentos durante o processo de padronização dos métodos laboratoriais utilizados, pelas colaborações técnicas e estatísticas, fundamentais para o trabalho.

- A CAPES e a FAMERP, pelos recursos financeiros que foram imprescindíveis para a execução desse trabalho.

EPÍGRAFE

“Agradeço todas as dificuldades que enfrentei, não fosse por elas, eu não teria saído do lugar. As facilidades nos impedem de caminhar. Mesmo as críticas nos auxiliam muito.”

Chico Xavier

LISTA DE FIGURAS

Artigo1

Artigo2

LISTA DE TABELAS

Artigo1

Artigo2

LISTA DE ABREVIATURAS E SÍMBOLOS

AMA-1 antígeno de membrana apical 1

ANOVA Análise de variância

CD28 Cluster de diferenciação 28

CD80 Cluster de diferenciação 80

CD86 Cluster de diferenciação 86

CNV variação no número de cópias (do inglês Copy number variation)

CSP proteína circunsporozoíta (do inglês circumsporozoite protein)

CTLA-4 antígeno 4 associado ao linfócito T citotóxico (do inglês cytotoxic T lymphocyte-associated antigen-4)

DBP proteína de ligação ao antígeno Duffy (do inglês duffy-binding protein)

DO Densidade óptica

ELISA antígeno 4 associado ao linfócito T citotóxico

HLA Antígeno leucocitário humano (do inglês human leukocyte antigen)

ICOS co-estimulador induzível de células T (do inglês *inducible T-cell costimulatory*)

IgG Imunoglobulina G

IL-4 Interleucina 4

IPA Índice parasitário anual

INDEL inserção-deleção

MHC complexo principal de histocompatibilidade

MIAs Marcadores Informativos de Ancestralidade

MSP Proteína de superfície do merozoíto (do inglês merozoite surface protein)

PBS Tampão salino fosfatado (do inglês phosphate-buffered saline)

PCR reação em cadeia da polimerase

PvAMA-1 antígeno dde membrana apical 1 de *Plasmodium vivax*

RFLP Polimorfismo de comprimento de fragmentos de restrição (do inglês restriction fragment length polymorphism)

SNPs polimorfismos de base única

TCR receptor do linfócito T

Th1 Linfócito T auxiliador (helper) $CD4^+$ Th1

RESUMO

Introdução: O *Plasmodium vivax* é a causa mais comum de malária humana na região Amazônica brasileira. A imunidade celular requer sinais co-estimulatórios para iniciar ou inibir as respostas das células T. CTLA-4 é um receptor expressado por células T ativadas. O objetivo do estudo foi analisar dois SNPs do gene *CTLA4* em pacientes com malária por *P. vivax* e correlacionar com parasitemia e níveis IL-4. **Métodos:** Um total de 182 indivíduos com malária por *P. vivax* foram incluídos no estudo. O DNA foi extraído a partir de amostras de sangue de acordo com o procedimento padrão. O protocolo de PCR-RFLP foi utilizado para indetificar as frequências alélicas e genotípicas dos polimorfismos. A densidade parasitária em indivíduos infectados foram registrados e expressos pelo número de formas assexuadas de *P. vivax* por microlitro de sangue assumindo uma contagem de 100 campos microscópicos. A dosagem sérica da IL-4 foram detectadas por Milliplex Map kit (Human Cytokine Magnetic Bead Panel-HCTOMAG-60K) com análise no equipamento Magpix/Luminex®.As análises estatísticas foram realizadas utilizando o programa R v 2.11.1. **Resultados:** para o polimorfismo na posição -1577 G/A, o genótipo G/A teve maior frequência (49.4%), seguido pelo genótipo G/G (41%) e o genótipo A/A (9.6%). Para o polimorfismo na posição -1722 T/C, o genótipo T/T foi o mais frequente (86.7%), seguido pelo genótipo T/C (12.3%) e o menos frequente o genótipo C/C (1%). Os níveis de IL-4 variaram entre 0,61 e 9,32 pg/mL. Não houve diferenças estatisticamente significantes tanto na parasitemia e níveis de IL-4 entre indivíduos com diferentes genótipos. **Conclusão:** O estudo também destaca a importância da realização de estudos de associação genética em diferentes populações étnicas. Os SNPs do gene *CTLA4* podem estar associados à

malária *vivax* em outras áreas endêmicas ou a outras doenças parasitárias, mas parecem não ter tal efeito nesta população.

Palavras-chave: Malária, *Plasmodium vivax*: polimorfismos de base única, *CTLA4*

ABSTRACT

Introduction: *Plasmodium vivax* is the most common cause of human malarial parasite infection in the Brazilian Amazon region. Cell-mediated immunity requires costimulatory activity to initiate or inhibit antigen-specific T-cell responses. Cytotoxic T lymphocyte-associated antigen (CTLA)-4 is an inhibitory receptor expressed by activated and regulatory T cells. The aim of this study was to analyze two coding single-nucleotide polymorphisms (SNPs) in *CTLA4* in patients with *P. vivax* malaria and their correlation with parasitaemia and plasma interleukin (IL)-4 levels. Methods: A total of 182 *P. vivax* malaria patients were enrolled in the study. DNA was extracted from blood samples using a standard procedure. A PCR-RFLP protocol was used to identify the genotype and allele frequencies of the 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. Serum levels of IL-4 were detected using a Milliplex Map kit (Human Cytokine/Chemokine Magnetic Bead Panel–HCYTOMAG-60K) and a Magpix/Luminex®. Analyses were performed using R version 2.8.1 statistical software. Results: 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%), and the least frequent was 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 in either parasitaemia or plasma IL-4 levels among individuals with different genotypes. Conclusion: The study also highlights the importance of conducting genetic association studies in different ethnic populations.

CTLA4 SNPs may be associated with malaria vivax in other endemic areas and other parasitic diseases, but they appear to have no such effect in this studied population.

Keywords: Malaria, *Plasmodium vivax*, Single nucleotide polymorphism, *CTLA4*

1. INTRODUÇÃO

1. Introdução

1.1 Malária – Considerações gerais

A malária é uma doença parasitária infecciosa causada por protozoários do gênero *Plasmodium,* da família Plasmodiidae, filo Apicomplexa, aproximadamente 120 espécies de *Plasmodium* que infectam aves, répteis e mamíferos já foram descritas[(1)], cinco dessas são reconhecidas como causa da malária humana; *Plasmodium falciparum, P. vivax, P. malariae* e o *P. ovale* e, recentemente, o *P. knowlesi* que originalmente acreditava-se que fosse parasita exclusivo de macacos, foi detectado na Malásia infectando humanos [(2,3)]. Essa doença é dos problemas mais graves de saúde pública no mundo, embora a grande maioria dos casos ocorra no continente africano, a doença encontra-se amplamente distribuída na América Latina, Sudeste Asiático e Oceania, estima-se que 3,3 bilhões de pessoas vivem em áreas de risco de transmissão de malária em 106 países e territórios[(2)].

Segundo a Organização Mundial de Saúde, ocorreram 207 milhões de casos de malária em 2012, resultando em aproximadamente 627 mil mortes, principalmente crianças menores de cinco anos. Nas Américas foram registrados 469 mil casos e o país com o maior número de casos notificados foi o Brasil com 52%, causadas por três espécies, *P. vivax, P. falciparum e P. malariae*, sendo o primeiro a espécie mais prevalente, responsável por 203.018 casos em 2012, 85% dos casos notificados[(2,4)] .

A doença é principalmente transmitida ao homem pela picada da fêmea do mosquito do gênero *Anopheles* infectada pelo *plasmodium*, durante o repasto sanguíneo no homem injeta esporozoítos na corrente sanguínea. Após a picada, os esporozoítos

migram e invadem as células do fígado (hepatócitos), dando início ao ciclo pré-eritrocítico ou esquizogonia tecidual. O *P. vivax* apresenta características biológicas distintas, como o desenvolvimento lento de alguns dos seus esporozoítos no fígado, dando origem a formas latentes ou hipnozoítos. A ativação dos hipnozoítos pode ocorrer semanas, meses ou anos mais tarde e, é responsável pela recaída da doença, permitindo o aparecimento do quadro clínico e condição potencial para a transmissão de gametócitos[5].

Os parasitos que estão na fase hepática, sofrem diferenciação e multiplicação assexuada e após alguns dias surgem os merozoítos que rompem as células do fígado que invadem os eritrócitos, nessa fase o *P. vivax* tem como alvo as eritrócitos jovens (reticulócitos), o *P. vivax* é capaz de invadir somente reticulócitos que expressam em sua superfície o determinante antigênico do grupo sanguíneo Duffy, um antígeno receptor de quimiocinas, o qual é o receptor da molécula ligante PvDBP. Indivíduos Duffy-negativos parecem ser completamente refratários a esta infecção [6], embora evidências recentes na África[7,8] e no Brasil[9] sugerem que indivíduos Duffy negativos possam se infectar com este protozoário, provavelmente por meio de outros receptores envolvidos no reconhecimento eritrocitário[8].

No ciclo eritrocítico, o parasito sofre uma série de transformações morfológicas, e desenvolvem para trofozoítos jovens (forma de anel) e posteriormente para trofozoítos maduros, no qual ocorre divisões para formação dos esquizontes e após a lise dos eritrócitos ocorre a liberação de novas formas merozoítas aptas a infectar outros eritrócitos. Depois de algumas gerações de merozoítos sanguíneos, ocorre diferenciação em estágios sexuados, os gametócitos masculinos e femininos, que são formas infectantes para o vetor. Durante um novo repasto sanguíneo no homem, o mosquito

não infectado ingere os gametócitos masculinos e femininos, ocorrendo o processo de fertilização entre os gametas, originado o oocineto que migra a parede intestinal, transforma-se em oocisto, multiplicando-se por esporogonia dando origem a milhares de esporozoítas, que migram para glândula salivar do mosquito onde amadurecem e se tornam capazes de infectar o hospedeiro vertebrado [5].

O ciclo biológico da malária é extremamente complexo e requer expressão de proteínas especializadas do hospedeiro para garantir a sobrevivência do parasito e a sua invasão em diversos tipos celulares [1]. A interação entre o esporozoíto e as células hepáticas ocorre através de uma proteína denominada circumsporozoíta (CSP), para a invasão dos eritrócitos várias moléculas do parasito interagem com receptores na célula hospedeira, como a proteína de superfície do merozoíto (MSP), o antígeno de membrana apical 1 (AMA-1), e a proteína de ligação ao antígeno Duffy (DBP). Essas proteínas são altamente imunogênicas, tem sido demonstradas como importantes alvos para desenvolvimento de vacinas.

1.2 Resposta Imune na Malária

A resposta imune na malária atua contra as diferentes formas evolutivas do parasita, envolvendo mecanismos humorais e celulares [4,10]. Em áreas em que a malária é altamente endêmica, crianças menores de cinco anos são altamente susceptíveis a doença, sendo comum casos fatais, após a exposição ao parasita crianças acima de cinco anos e adultos, desenvolvem uma proteção naturalmente adquirida, apresentando baixa parasitemia e ausência de sintomatologia. A intensidade destas manifestações depende

da idade do indivíduo e de exposições prévias ao parasita, desaparece se o contato com o parasito for cessado. Além disto, a imunidade à malária é caracterizada por ser espécie e estágio-específica[11].

O desenvolvimento de uma resposta imune depende de uma rede complexa de células, mas no centro do processo está o papel dos linfócitos T ativados. Para que ocorra a ativação e proliferação dos linfócitos T, são necessários dois sinais independentes, o primeiro ocorre pela ligação do receptor do linfócito T (TCR) a um peptídeo antigênico apresentado pelo complexo principal de histocompatibilidade (MHC) presente nas células apresentadoras de antígenos[12,13]. O segundo sinal co-estimulatório é gerado pela interação de moléculas, B7.1 ou CD80 e B7.2 ou CD86,[14] e a molécula CD28, expresso constitutivamente na superfície das células T.[12,13] Por outro lado, o sinal co-inibitório diminuem este tipo de resposta promovendo a manutenção da homeostase imunológica, como o CTLA-4 (do inglês *cytotoxic T lymphocyte-associated antigen 4*).[13,15]

A molécula CTLA-4 é expresso na superfície das células T ativadas e possui os mesmos ligantes que o CD28.[13] A interação da CTLA-4 com CD80 e CD86 acarreta em uma regulação negativa para resposta imunológica, com transmissão de um sinal que causa anergia clonal, tolerância ou desativação celular,[13, 15, 16] além da inibição da produção de citocinas e da progressão do ciclo celular.[17]

O gene *CTLA4* está localizado na região cromossômica 2q33, este gene possui 7195 kilobases (kb) e apresenta 4 éxons. Polimorfismos de base única (SNPs) na região promotora desse gene podem potencialmente afetar a expressão do mesmo por meio de alteração da transcrição e tem sido estudados em doenças infecciosas, auto-imunes e neoplasias[13]. Alguns estudos demonstram que polimorfismos nos genes *CTLA4* e

CD28 conferem susceptibilidade e predisposição genética às doenças autoimunes.[18] O bloqueio do *CTLA4* aumenta a resistência ao *Trypanosoma cruzi,*[19] e também para *Leishmania donovani.*[20] Na malária, estudos experimentais com *Plasmodium berghei,* demonstram que o bloqueio do gene *CTLA4* conduziu a um aumento exacerbado nos casos de malária cerebral.[21] Além disso, essa molécula contribui na regulação para o equilíbrio Th1/Th2.[22]

A Interleucina 4 (IL4) é uma citocina pertencente à família das hematopoietinas, produzidas pelas células T e mastócitos. A proteína expressa atua na proliferação e diferenciação de células B para posterior produção de anticorpos, com diminuição da resposta tipo Th1, além de estimular o crescimento e diferenciação de eosinófilos.[23] Nas células B ativadas estimula a síntese principalmente de IgE e de IgG de classe 1, sendo seu efeito modulado negativamente pelo interferon gama.[24]

O antígeno de membrana apical 1 (AMA-1) é uma proteína transmembrana altamente conservada, presente em todas as espécies de *Plasmodium,* a estrutura da AMA-1 é formada por um ectodomínio rico em cisteínas, uma região transmembrana e uma região C-terminal, é uma proteína de 83 kDa está presente inicialmente nos micronemas e posteriormente na superfície do merozoíto durante a invasão nos eritrócitos.[25, 26] A função desempenhada por AMA-1 não é bem caracterizada, porém evidências demonstram o papel essencial dessa proteína durante o processo de invasão dos merozoítos aos eritrócitos, sendo um dos principais alvos de estudos para vacina contra a malária, por ser altamente imunogênica e os níveis de anticorpos contra AMA-1 serem importantes para o bloqueio dessa invasão,[27] sendo capazes de reduzir a multiplicação do parasita e proteger contra infecções.[28]

1.3 Estudos de Associação e Ancestralidade

Estudos de associação são amplamente utilizados para identificar genes relacionados à determinada doença, estudos do tipo caso-controle constituem uma importante ferramenta para mapear esses genes e verificam diferenças na frequência de polimorfismos entre os indivíduos que apresentam determinado fenótipo (caso) com indivíduos que não o apresentam (controle). Entretanto, nesse tipo de estudo, existe o risco obter resultados falso-positivos ou associações espúrias. Geralmente associações espúrias são decorrentes da estratificação populacional, que ocorre em grupos que possuem frequências alélicas diferentes entre e dentro dos subgrupos ou em populações miscigenadas com diferentes frações de ancestralidade. [29]

Dessa forma, nos estudos de associação deve-se considerar a análise de ancestralidade para eliminar a possibilidade de a associação ser consequência do *background* genético da população estudada. Com este intuito, marcadores genéticos que capturem diferenças nas frequências alélicas entre populações continentais que possam atribuir a ancestralidade de um indivíduo, ou ainda estimar proporções de populações parentais em populações miscigenadas, são especialmente informativos para se estimar a miscigenação e são conhecidos como marcadores informativos de ancestralidade e (AIMs, do inglês *ancestry informative markers*). Dentre os diferentes marcadores genéticos (variação no número de cópias (CNV), inserção-deleção (INDEL), microssatélites), os mais empregados em estudos de associação são do tipo SNP, que consiste na alteração de um único nucleotídeo por outro.

Esta observação é de grande relevância quando se pretende realizar estudos em populações miscigenadas como a brasileira. Geralmente, indicadores de aparência

física, especialmente a cor da pele, são utilizados para determinar a ancestralidade. Entretanto, como consequência da intensa miscigenação da população brasileira, resultado de cinco séculos de cruzamentos interétnicos entre europeus, africanos e ameríndios, estes indicadores são pobres descritores da ancestralidade genômica.[30, 31, 32] Dessa forma, faz-se necessário a utilização de AIMs ao se realizar estudos de associação na população brasileira.

1.4 Objetivos

Geral

Investigar a influência de polimorfismo no gene CTLA-4 na malária vivax em uma área endêmica da Amazônia brasileira

Objetivos específicos:

1. Avaliar se a ancestralidade genética influência na distribuição de polimorfismos no gene *CTLA-4*,
2. Estimar as frequências alélicas e genotípicas de polimorfismos no gene *CTLA4* de amostras de pacientes infectados por *P. vivax*,
3. Correlacionar estas frequências com níveis de IL-4, anticorpos contra AMA-1 e parasitemia, e outros fatores como gênero, idade, período de exposição, episódios prévios de malária, primo-infectados, ancestralidade genética.

2. MATERIAL E MÉTODOS

2. Material e Métodos

Cenário do estudo e da população

A amostra foi constituída por 182 indivíduos infectados pelo *P. vivax*, coletadas no município de Goianésia do Pará (03º50'33" S; 49º05'49" W), este município está entre os cinco que concentram 50% dos casos de malária do Estado do Pará. Os pacientes incluídos nesse estudo (n=182) preencheram os seguintes critérios: apresentação aos cuidados médicos por causa por causa dos sintomas clínicos de malária, idade >14 anos de idade, e com diagnóstico positivo por gota espessa para *P. vivax*. Todos os indivíduos assinaram o termo de consentimento livre e esclarecido. O protocolo deste estudo foi analisado e aprovado (Processo número 01774812.2.0000.5415) pelo Conselho de Ética em Pesquisa da Faculdade de Medicina de São José do Rio Preto, Estado de São Paulo, Brasil, e pelas autoridades de saúde do município de Goianésia do Pará. Amostras de sangue foram armazenadas à - 20^{o}C.

Para determinação da ancestralidade: A amostra foi constituída de 266 indivíduos não relacionados, sendo 175 homens e 91 mulheres, provenientes do município de Goianésia do Pará. O projeto foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Medicina de São José do Rio Preto (FAMERP 4599/2011).

Determinação da parasitemia

Os resultados da Gota espessa foram confirmados por microscopistas experientes independentes que desconheciam cada resultado de acordo com os procedimentos recomendados pela Organização Mundial de Saúde. A densidade parasitária em indivíduos infectados foram registrados e expressos pelo número de formas assexuadas de *P. vivax* por microlitro de sangue assumindo uma contagem de 100 campos microscópicos. Os resultados da Gota Espessa foram confirmados por semi-nested PCR com base no protocolo descrito por Snounou et al. (1993)[33].

Genotipagem

Os DNAs genômicos do sangue periférico foram extraídos utilizando o kit de extração Easy-DNATM (Invitrogen, Carlsbad, CA, USA). A identificação dos genótipos para os polimorfismos de nucleotídeo único (SNPs) foram realizadas por PCR-RFLP. Para o genótipo na posição -1577 G>A do gene *CTLA4* foram utilizados os oligonucleotídeos iniciadores 5' CTTCATGCCGTTTCCAACTT 3' e 5' ATCTCCTCCAGGAAGCCTCTT 3'. A reação da PCR, de 25 uL foi composta com: Tampão 1X (Tris-HCl pH 8,4 200 mM, KCl 500 mM), $MgCl_2$ 1,5 mM, 0,2 mM de cada dNTP, 0,6 µM de cada iniciador, 0,5 U de Platinum Taq DNA Polimerase (Invitrogen Life Techonologies®, Brasil) e 1 µL de DNA genômico, gerando um fragmento de 536pb em gel de agarose 1,5%. A reação de amplificação consistiu de uma desnaturação inicial por 94ºC por 5 min seguido de 35 ciclos de 94ºC por 30 s, 52ºC for 30 s, e 72ºC para 1 min e finalmente um de ciclo de 72ºC por 5 min. O produto amplificado foi digerido a enzima de restrição *MboII* (Fermentas®) a 37ºC por 20 minutos e visualizado

em gel de agarose 2% corado com gel red. Os genótipos atribuídos foram baseados no tamanho dos fragmentos resultantes:442, 59, 26 e 9 pb para o alelo *G* e fragmentos de 275, 167, 59, 26 e 9 pb correspondentes ao alelo A.

Para o SNP na posição -1722 T>C no gene *CTLA4* foram utilizados os oligonucleotídeos iniciadores 5' CTTCATGCCGTTTCCAACTT 3' e 5' CCTTTTCTGACCTGCCTGTT 3'. A reação de PCR, de 25 uL foi composta com: Tampão 1X (Tris-HCl pH 8,4 200 mM, KCl 500 mM), $MgCl_2$ 1,5 mM, 0,2 mM de cada dNTP, 0,6 µM de cada iniciador, 0,5 U de Platinum Taq DNA Polimerase e 1 µL de DNA genômico, gerando um fragmento de 400 pb em gel de agarose 1,5%. A PCR consistiu de uma desnaturação inicial por 94ºC por 5 min seguido de 35 ciclos de 94ºC por 30 s, 52ºC for 30 s, e 72ºC para 1 min e finalmente um de ciclo de 72ºC por 5 min. O produto amplificado foi digerido com a enzima de restrição *BbvI* (Fermentas®) a 37ºC overnight e visualizado em gel de agarose 2% corado com gel red. A identificação dos genótipos foi baseada no tamanho dos fragmentos resultantes: 400 pb correspondendo ao alelo T e fragmentos de 210 e 190 pb para o alelo C.

Para determinação da ancetralidade: Foi realizada a identificação dos seguintes SNPs, por PCR-RFLP: *-372G>A* (rs35593994) e *+17T>C* (rs3116496) no gene *CD28*, *-318C>T* (rs5742909) e *+49A>G* (rs231775) no gene *CTLA4*, *+1564T>C* (rs4675378) no gene *ICOS*, *+1057G>A* (rs1129055) no gene *CD86*, *-726T>C* (rs3092945) no gene *CD40L*, *-1C>T* (rs1883832) no gene *CD40* e *-871C>T* (rs9514828) no gene *BLYS*. Todas as reações de PCR foram realizadas em um volume final de 25 µL contendo Tampão 1X (Tris-HCl 20 mM pH 8,4, KCl 50 mM), $MgCl_2$ 1,5 mM, 0,2 mM de cada dNTP (dATP, dTTP, dCTP, dGTP), 0,4 pmol de cada iniciador e 0,5 U de Platinum Taq DNA Polimerase (Invitrogen, São Paulo, Brasil). As amplificações foram feitas no

termociclador DNA MasterCycler (Eppendorf, Hamburgo, Alemanha), nas seguintes condições: uma etapa inicial de 5 minutos a 94ºC, 35 ciclos de 30 segundos a 94ºC, 30 segundos em uma temperatura de anelamento de acordo com o iniciador utilizado e 1 minuto a 72ºC, e uma etapa final de 10 minutos a 72ºC. As sequências dos iniciadores, bem como as temperaturas de anelamento utilizadas em cada reação estão descritas na tabela 1. Os produtos da amplificação foram visualizados em gel de agarose 2% corado com GelRed™ (Biotium, Hayward, EUA) e foram submetidos à digestão utilizando enzimas da marca Fermentas (Vilnius, Lituânia), e os protocolos seguiram as recomendações do fabricante. As enzimas utilizadas, bem como o tamanho dos fragmentos resultantes da digestão de cada polimorfismo estão apresentadas na tabela 1. Os produtos da digestão foram visualizados em gel de agarose 2,5% corado com GelRed™ (Biotium, Hayward, EUA), com exceção dos polimorfismos *CD40 -1C>T* e *CTLA4 -318C>T*, que foram visualizados em gel de poliacrilamida 12,5%, corado com brometo de etídio.

Ensaio de níveis de citocina IL-4

A dosagem sérica da IL-4 foi realizada segundo o protocolo do kit (Milliplex Map kit, Human Cytokine Magnetic Bead Panel- HCTOMAG-60K) com análise no equipamento Magpix/Luminex®. As amostras de soro dos pacientes com malária estavam congeladas à temperatura -20°C e foram descongeladas uma única vez para a realização do ensaio. O princípio desse teste é pela detecção da IL-4, por meio de captura, por um anticorpo anti-IL-4, fixado em beads (microesferas de poliestireno), em um ensaio sanduíche. O software xPONENT analisa as imagens

(http://www.gendiag.com.br; http://www.luminexcorp.com). As dosagens foram realizadas em duplicata e os valores expressos em média do nível do analito.

Avaliação da resposta de anticorpos contra AMA-1

A avaliação de anticorpos IgG total contra a PvAMA-1 foi realizada utilizando a proteína recombinante representando o ectodomínio da proteína (aminoácidos 43 a 487) do *P. vivax* (cepa Belém) expressa em *Pichia pastoris*, de acordo com o protocolo descrito por Vicentin et al., 2014[34].

Os 96 poços das placas de ELISA (Costar, Corning Inc., NY, EUA) foram sensibilizados com 50 µL da proteína PvAMA-1 (2 µg/mL) diluída em tampão carbonato 0,05 M pH 9,0, por 16 horas a temperatura ambiente. Posteriormente, as placas foram lavadas com PBS Tween 0,05% e bloqueadas com 200 µL de PBS leite 5%. Após 2 horas a 37°C, as placas foram lavadas com PBS Tween 0,05% e 50 µL do plasma, diluído 1:100 em PBS leite 5%, foram adicionados em cada poço, em duplicata, e as placas foram incubadas por 2 horas a temperatura ambiente. Após nova lavagem das placas com PBS Tween 0,05%, foram adicionados, em cada poço, 50 µL de conjugado anti-IgG humano ligado a peroxidase (Sigma 0170, MO, EUA), diluído 1:5.000 em PBS leite 5%, e as placas foram incubadas por 1 hora em temperatura ambiente. Em seguida, após nova lavagem com PBS Tween 0,05%, as placas foram reveladas com a adição de 100 µL de OPD (1 mg/mL) diluído em tampão fosfato-citrato pH 5,0 contendo 0,03% de peróxido de hidrogênio. Essa reação foi mantida no escuro e

interrompida após 10 minutos pela adição de 50 uL de H_2SO_4 4N em cada poço. A densidade ótica (OD) foi quantificada em leitora de ELISA (Awareness Technology, Stat Fax 2100, FL, EUA) em um comprimento de onda de 492 nm. O ponto de corte foi estabelecido pela média das ODs de 40 amostras de plasma de indivíduos sem histórico de malária, residentes em São José do Rio Preto, interior do Estado de São Paulo (área não endêmica de malária) acrescida de três desvios padrões.

Determinação da ancestralidade

A genotipagem para a determinação da ancestralidade foi realizada utilizando 48 marcadores do tipo INDEL, previamente padronizados e validados.[35] Os marcadores foram selecionados seguindo dois critérios principais: grandes diferenças nas frequências alélicas entre africanos, europeus e/ ou ameríndios (≥ 40%) e estarem localizados em diferentes cromossomos ou em regiões físicas distantes quando no mesmo cromossomo. As reações de PCR foram realizadas em três sistemas *multiplex*, cada um contendo 16 pares de iniciadores marcados com fluorescência. A eletroforese foi realizada em sequenciador automático *ABI PRISM 3130 Genetic Analyzer* (Applied Biosystems). As sequências dos iniciadores, bem como as condições de ciclagem e da eletroforese capilar estão descritas em Santos et al (2010). Para estimar a mistura interétnica individual, foi utilizado o programa *Structure* versão 2.3.4 (http://pritch.bsd.uchicago.edu/software.html).

Análises Estatísticas

Toda a análise estatística foi realizada com o programa R v 2.11.1 (http://www.r-project.org). As frequências genotípicas e alélicas para cada variante foram obtidas por meio do pacote *genetics*.[36] Utilizando este pacote, foram avaliados desvios do Equilíbrio de Hardy-Weinberg. O teste não paramétrico de Kruskal-Wallis foi utilizado para testar diferenças na parasitemia e níveis de anticorpos em relação aos genótipos. Para avaliar diferenças nas médias dos níveis de IL-4 em relação aos genótipos, foi empregado o teste de variância (ANOVA). A análise entre os níveis de IL-4 e parasitemia foi realizada através da correlação de Spearman. As frequências haplotípicas foram estimadas pelo método da máxima verossimilhança que utiliza o algoritmo EM (do inglês *expectation-maximization*), que faz parte do pacote *haplo.stats*.[37] A análise de variância (ANOVA e teste t de Student) foi utilizada para testar as diferenças nas proporções de cada uma das ancestralidades entre os diferentes genótipos. Um modelo de regressão logística binária foi desenvolvido para explorar graficamente a associação dos polimorfismos com a ancestralidade individual estimada, utilizando o pacote *ggplot2* (Wickham 2009). Todos os pacotes estão implementados no programa R versão 2.11.1 (http://www.r-project.org). Valores de $p<0,05$ foram considerados estatisticamente significantes.

3. RESULTADOS

3 Resultados

Artigo 1 (Principal)

Título: *CTLA4* gene polymorphisms (-1577 G/A and -1722 T C) versus serum IL-4 levels, humoral immune responses to PvAMA-1 and parasitemia in individuals with *Plasmodium vivax* malaria in the Brazilian Amazon region.

Autores: Pamella C. A. Trindade, Gustavo C. Cassiano, Franciele M. M. B. Tomaz, Adriana A. C. Furini, Marcela P. Capobianco, Marinete M. Póvoa, Carlos E. Cavasini, Valéria D. Fraga, Luciana M. Conceição, Sônia M. Oliani, Lucas R. Azevedo, Ricardo L. D. Machado.

Periódico: Submetido à revista Acta Tropica.

Artigo 2

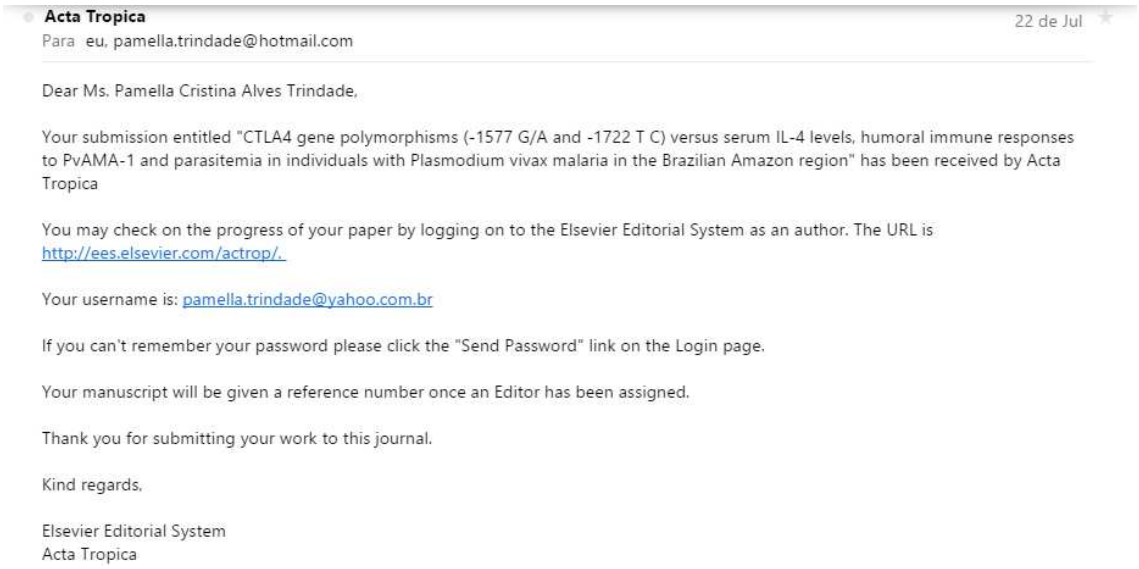
Título: Impact of population admixture on the distribution of immune response co-stimulatory genes polymorphisms in a Brazilian population

Autores: Gustavo C. Cassiano, Eduardo J. M. Santos, Maria H. T. Maia, Adriana A. C. Furini, Luciane M. Storti-Melo, Franciele M. B. Tomaz, Pamella C. A. Trindade, Marcela P. Capobianco, Marcos A. T. Amador, Giselle M. R. Viana, Marinete M. Póvoa, Sidney E. B. Santos[e], Ricardo L. D. Machado.

Periódico: Submetido à revista Human Immunology

ARTIGO 1

Artigo 1 (Principal)



Acta Tropica 22 de Jul

Para eu, pamella.trindade@hotmail.com

Dear Ms. Pamella Cristina Alves Trindade,

Your submission entitled "CTLA4 gene polymorphisms (-1577 G/A and -1722 T C) versus serum IL-4 levels, humoral immune responses to PvAMA-1 and parasitemia in individuals with Plasmodium vivax malaria in the Brazilian Amazon region" has been received by Acta Tropica

You may check on the progress of your paper by logging on to the Elsevier Editorial System as an author. The URL is http://ees.elsevier.com/actrop/.

Your username is: pamella.trindade@yahoo.com.br

If you can't remember your password please click the "Send Password" link on the Login page.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards.

Elsevier Editorial System
Acta Tropica

CTLA4 gene polymorphisms (-1577 G/A and -1722 T C) versus serum IL-4 levels, humoral immune responses to PvAMA-1 and parasitemia in individuals with *Plasmodium vivax* malaria in the Brazilian Amazon region

TRINDADE, P.C.A.[a]*, CASSIANO, G.C.[a, b], TOMAZ, F.M.M.B. [a], FURINI, A.A.C.[a], CAPOBIANCO, M.P.[a, b], PÓVOA, M.M.[c], SANTOS, S.E.B.[d], CAVASINI, C.E.[a], FRAGA, V.D.[a], CONCEIÇÃO, L.M.[a], OLIANI, S.M.[b], AZEVEDO, L.R.[b], MACHADO, R.L.D.[a, b, c].

[a]Medical School of São José do Rio Preto (Faculdade de Medicina de São José do Rio Preto - FAMERP), Centro de Investigação de Microrganismos, Departamento de Doenças Dermatológicas, Infecciosas e Parasitárias, Av. Brigadeiro Faria Lima 5416, 15090-000, São José do Rio Preto, São Paulo, Brazil.

[b]São Paulo State University (Universidade Estadual Paulista - Unesp), "Júlio Mesquita Filho", Rua Cristóvão Colombo 2265, 15054-000, São José do Rio Preto, São Paulo, Brazil.

[c]Evandro Chagas Institute (Instituto Evandro Chagas - IEC),Laboratório em Pesquisa Básica de Malária, Rodovia BR316, Km 7, s/n, Ananindeua, Pará, Brazil.

[d]Federal University of Pará (Universidade Federal do Pará - UFPA), Rua: Augusto Corrêa, 01, 66075-110, Belém, Pará, Brazil.

*Corresponding Author: Faculdade de Medicina de São José do Rio Preto, Centro de Investigação, Departamento de Doenças Dermatológicas, Infecciosas e Parasitárias.

Avenida Brigadeiro Faria Lima, 5416, 15090-000, São José do Rio Preto, SP, Brasil, Tel.: +55 17 32015736.

E-mail address: pamella.trindade@yahoo.com.br (P. C. A. TRINDADE)

Abstract

Introduction: *Plasmodium vivax* is the most common cause of human malarial parasite infection in the Brazilian Amazon region. Cell-mediated immunity requires costimulatory activity to initiate or inhibit antigen-specific T-cell responses. Cytotoxic T lymphocyte-associated antigen (CTLA)-4 is an inhibitory receptor expressed by activated and regulatory T cells. The aim of this study was to analyze two coding single-nucleotide polymorphisms (SNPs) in *CTLA4* in patients with *P. vivax* malaria and their correlation with parasitaemia and plasma interleukin (IL)-4 levels. Methods: A total of 182 *P. vivax* malaria patients were enrolled in the study. DNA was extracted from blood samples using a standard procedure. A PCR-RFLP protocol was used to analyze the genotype and allele frequencies of the 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. Serum levels of IL-4 were detected using a Milliplex Map kit (Human Cytokine/Chemokine Magnetic Bead Panel–HCYTOMAG-60K) and a Magpix/Luminex®. Analyses were performed using R version 2.8.1 statistical software. Results: 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%), and the least frequent was

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 in either parasitaemia or plasma IL-4 levels among individuals with different genotypes. Conclusion: *CTLA4* SNPs may be associated with malaria vivax in other endemic areas and other parasitic diseases, but they appear to have no such effect in this studied population. The study also highlights the importance of conducting genetic association studies in different ethnic populations.

Keywords: Malaria, *Plasmodium vivax*, Single nucleotide polymorphism, *CTLA4*

1. Introduction

Malaria is a major cause of morbidity and mortality for millions of people worldwide, mainly in tropical and subtropical areas (Tangpukdee et al., 2009). *Plasmodium vivax* is the predominant species in Brazil and was responsible for 203,018 cases in 2012, i.e., 85% of reported cases (WHO, 2013).

The immune response in malaria involves humoral and cellular mechanisms, and it is directly related to disease pathophysiology and hence to its signs and symptoms (Medina et al., 2011; Oliveira-Ferreira et al., 2010). For the immune response to occur, interactions between T-cell antigen receptors (TCR) and CD4 or CD8 co-receptors that bind to human leukocyte antigen (HLA) are necessary to initiate T cell proliferation (Pincerati et al., 2010; Rummel et al., 2004). However, this binding does not result in clonal expansion, as this process requires costimulatory molecules B7.1 or CD80 and B7.2 or CD86 (Elias et al., 2005) and their T cell receptor, CD28, which is constitutively expressed on the cell surface (Pincerati et al., 2010; Rummel et al., 2004). Moreover, co-inhibitory molecules, such as CTLA-4 (cytotoxic T lymphocyte-

associated antigen-4), also participate in this process (Liu and Zhang, 2013; Pincerati et al., 2010).

CTLA-4 is expressed on the surface of activated T cells and has the same ligands as *CD28* (Pincerati et al., 2010). The interaction of CTLA-4 with CD80 and CD86 leads to a downregulation of the immune response by transmitting a signal that causes clonal anergy, tolerance or cell deactivation (Liu and Zhang, 2013; Pincerati et al., 2010; Rudd and Schneider, 2003), in addition to inhibiting cytokine production and progression of the cell cycle (Sayegh, 1999). Some studies have demonstrated that polymorphisms in the *CTLA4* and *CD28* genes confer susceptibility and genetic predisposition to autoimmune diseases (Cheng et al., 2006). *CTLA4* blockade increases resistance to *Trypanosoma cruzi* (Martins et al., 2004) and also to *Leishmania donovani* (Murphy et al. 1998). With regard to malaria, experimental studies with *P. berghei* showed that *CTLA4* gene blockade led to a considerable increase in the number of cerebral malaria cases (Jacobs et al., 2002). Furthermore, this molecule contributes to regulation of the Th1/Th2 balance (Masteller et al., 2000). Interleukin 4 (IL-4) is a cytokine produced mainly by Th2 cells and mast cells that acts in the proliferation and differentiation of B cells for subsequent production of antibodies (Jha et al., 2012). *CTLA4* blockade during *Nippostrongylus brasiliensis* infection resulted in high production of Th2 cytokines, such as IL-4, and a decrease in this parasite (McCoy et al., 1997).

Apical membrane antigen 1 (AMA-1) is a protein synthesized by mature stages of the parasite and is present on the surface of the merozoite during the invasion of erythrocytes (Arévalo-Herrera et al., 2010; Bueno et al., 2011). AMA-1 is one of the main targets of studies for a malaria vaccine because it is highly immunogenic and

because anti-AMA-1 antibodies are important for blocking this invasion (Zakeri et al., 2013), as they are able to reduce the replication of the parasite and protect against infections (Remarque et al., 2008).

The *CTLA4* gene is located in the 2q33 chromosome region. Single nucleotide polymorphisms (SNPs) in the promoter region of this gene may potentially affect its expression through changes in gene transcription and have been studied in infectious and autoimmune diseases as well as in cancer (Pincerati et al., 2010). Based on these data, we studied the allele and genotype frequencies of *CTLA4* gene polymorphisms at positions -1577 G>A (rs11571316) and -1722 T>C (rs733618) and their correlation with levels of parasitemia, IL-4 and anti-AMA-1 antibodies in patients infected with *P. vivax*.

2. Materials and Methods

2.1 Study and population characteristics

The sample consisted of 182 individuals infected with *P. vivax* and was collected in the municipality of Goianésia do Pará (03º50'33" S; 49º05'49" W), which is located in the southeastern Pará mesoregion and in the microregion of Paragominas, and it borders the municipalities of Breu Branco, Novo Repartimento, Dom Eliseu, Ipixuna do Pará, Jacundá and Rondon do Pará. The municipality has a land area of 7,021 km^2 with an estimated population of 29,161 inhabitants, of whom 52% are over 14 years old. The population density is 4.5 inhabitants per km^2, and the municipality is approximately 350 km from the capital. This municipality is among the five that contain 50% of the malaria cases in the State of Pará. In total, 1,136 cases of malaria were reported in 2012, of which 80% corresponded to cases of *P. vivax* infection. Three locations (settlement

areas) were chosen: Santa Paula, Rouxinol and Ararandeua, all with high annual parasitic indices (API) in 2009, namely 368.4, 1484.4 and 134.4, respectively (SIVEP-Malária, n.d.). The patients included in the present study (n=182) met the following criteria: sought medical care because of clinical symptoms of malaria, were aged > 14 years old and had a positive diagnosis of *P. vivax* by thick blood smear. All individuals signed an informed consent form. Peripheral blood samples were obtained from individuals from the malaria endemic area of Goianésia do Pará, Brazilian Amazon region. The study protocol was reviewed and approved (Process number 01774812.2.0000.5415) by the Research Ethics Committee of the Medical School of São José do Rio Preto (Faculdade de Medicina de São José do Rio Preto – FAMERP), State of São Paulo, Brazil, and by the health authorities of the municipality of Goianésia do Pará. The blood samples were stored at -20°C.

2.2 Parasitemia determination

Thick blood smear results were confirmed by independent experienced microscopists who were blind to each result, according to the procedures recommended by the World Health Organization. The parasite density in infected individuals was recorded and expressed as the number of *P. vivax* asexual forms per microliter of blood when counting 100 microscopic fields. The thick blood smear results were confirmed by semi-nested PCR based on the protocol described by Snounou et al. (1993).

2.3 Estimates of interethnic admixture

The population of northern Brazil is highly mixed and formed mainly by crosses between Europeans, Africans, and Native Americans. To avoid spurious interpretations

resulting from population substructure, we used a panel of 48 ancestry informative markers (AIMs) to estimate the proportion of individual interethnic admixture in our sample, following a previously described protocol. The Structure software version 2.3.4 was used, and three parental populations (European, African, and Native Americans) were assumed as described by Santos et al. (Santos et al., 2010). These estimates were used as covariates in the multivariate analyses to adjust for population stratification.

2.4 Genotyping

Genomic DNA samples from the peripheral blood of all individuals included in the present study were extracted using the Easy-DNATM extraction kit (Invitrogen, Carlsbad, CA, USA). SNP genotyping was performed using PCR-RFLP. The primers 5' CTTCATGCCGTTTCCAACTT 3' and 5' ATCTCCTCCAGGAAGCCTCTT 3' were used for genotyping at position -1577 G>A of the *CTLA4* gene. The 25-µL PCR reaction consisted of the following: 1X buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.6 µM of each primer, 0.5 U Platinum Taq DNA polymerase (Invitrogen Life Technologies®, Brazil) and 1 µL of genomic DNA, generating a 536-bp fragment as visualized on a 1.5% agarose gel. The amplification reaction consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min and finally a 72°C cycle for 5 min. The amplified product was digested with the *MboII* restriction enzyme (Fermentas®) at 37°C for 20 minutes and visualized on a 2% agarose gel stained with GelRed. The genotypes were assigned based on the size of the resulting fragments: 442, 59, 26 and 9 bp for allele G and fragments of 275, 167, 59, 26 and 9 bp corresponding to allele A.

For SNPs at position -1722 T>C in the *CTLA4* gene, the primers 5' CTTCATGCCGTTTCCAACTT 3' and 5' CCTTTTCTGACCTGCCTGTT 3' were used. The 25-µL PCR reaction consisted of: 1X buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.6 µM of each primer, 0.5 U Platinum Taq DNA polymerase and 1 µL of genomic DNA, generating a 400-bp fragment as visualized on a 1.5% agarose gel. The amplification reaction consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min and finally one 72°C cycle for 5 min. The amplified product was digested with *BbvI* restriction enzyme (Fermentas®) at 37°C overnight and visualized on a 2% agarose gel stained with GelRed. Genotyping was performed based on the size of the resulting fragment: 400 bp corresponding to the T allele and fragments of 210 and 190 bp for allele C.

2.5 Cytokine assay for IL4 levels

Serum IL-4 levels were measured according to the kit protocol Milliplex MAP Human Cytokine/Chemokine Panel (# HCYTOMAG-60K, Millipore, Boston, MA, USA) with analysis on the Magpix/Luminex® device. Serum samples from malaria patients were frozen at -20°C and were thawed only once to perform the assay. The principle of this assay is that IL-4 is detected by capturing it using an anti-IL-4 antibody and fixing it on beads (polystyrene microspheres) in a sandwich immunoassay. The images were analyzed using xPONENT software. The measurements were performed in duplicate, and the values were expressed as mean analyte levels.

2.6 AMA-1 assessment of the serological response against *P. vivax*

The assessment of total IgG antibodies against PvAMA-1 was performed using a recombinant protein representing the ectodomain of the *P. vivax* ('Belém' strain) protein (amino acids 43 to 487) expressed in *Pichia pastoris* according to the protocol described by Vicentin et al. 2014.

The 96 wells of the ELISA plates (Costar, Corning Inc., NY, USA) were sensitized with 50 µL of the PvAMA-1 protein (2 µg/mL) diluted in 0.05 M carbonate buffer (pH 9.0) for 16 hours at room temperature. Subsequently, the plates were washed with 0.05% phosphate-buffered saline (PBS) Tween and blocked with 200 µL PBS with 5% milk. After 2 hours at 37°C, the plates were washed with 0.05% PBS Tween and 50 µL of plasma diluted 1:100 in PBS with 5% milk was added to each well in duplicate, after which the plates were incubated for 2 hours at room temperature. After re-washing the plates with 0.05% PBS Tween, 50 µL of anti-human IgG-peroxidase conjugate (Sigma 0170, MO, USA) diluted 1:5,000 in PBS with 5% milk was added to each well, and the plates were incubated for 1 hour at room temperature. After re-washing with 0.05% PBS Tween, the plates were developed by adding 100 µL of o-phenylenediamine (1 mg/mL) diluted in phosphate-citrate buffer (pH 5.0) containing 0.03% hydrogen peroxide. This mixture was left to react in the dark and stopped after 10 minutes by adding 50 µL of 4 N H_2SO_4 to each well. The optical density (OD) was measured with an ELISA reader (Awareness Technology Stat Fax 2100, FL, USA) at a wavelength of 492 nm. The cutoff point was established by using the mean of the ODs plus three standard deviations of 40 plasma samples from individuals with no history of malaria living in São José do Rio Preto in the countryside of the state of São Paulo, which is not a malaria endemic area.

2.7 Statistical Analyses

All statistical analyses were performed using R software v.2.11.1 (http://www.r-project.org). The genotype and allele frequencies for each variant were obtained through the *genetics* package (Warnes et al., 2011). Using this package, deviations from Hardy-Weinberg equilibrium were evaluated. The nonparametric Kruskal-Wallis test was used to test differences in parasitemia and in antibody levels according to genotypes. The analysis of variance (ANOVA) was used to evaluate differences in the mean IL-4 levels according to genotypes. The analysis between the levels of IL-4 and parasitemia was performed using Spearman's correlation. Differences in proportions were assessed using the Chi-square test, and p-values <0.05 were considered significant.

3. Results

The IL-4 levels varied between 0.61 and 9.32 pg/mL. The parasitemia levels as measured by thick blood smear ranged from 5 to 20,000 parasites/mm^3. As summarized in Figures 1a and 1b, there was no significant correlation between the levels of parasitemia and anti-AMA-1 antibodies (p=0.43 and rho=0.06) and between levels of parasitemia and IL-4 (p=0.48 and rho=-0.08). Of the 182 individuals analyzed in the present study, 124 were positive for IgG antibodies against PvAMA-1 and 58 were negative.

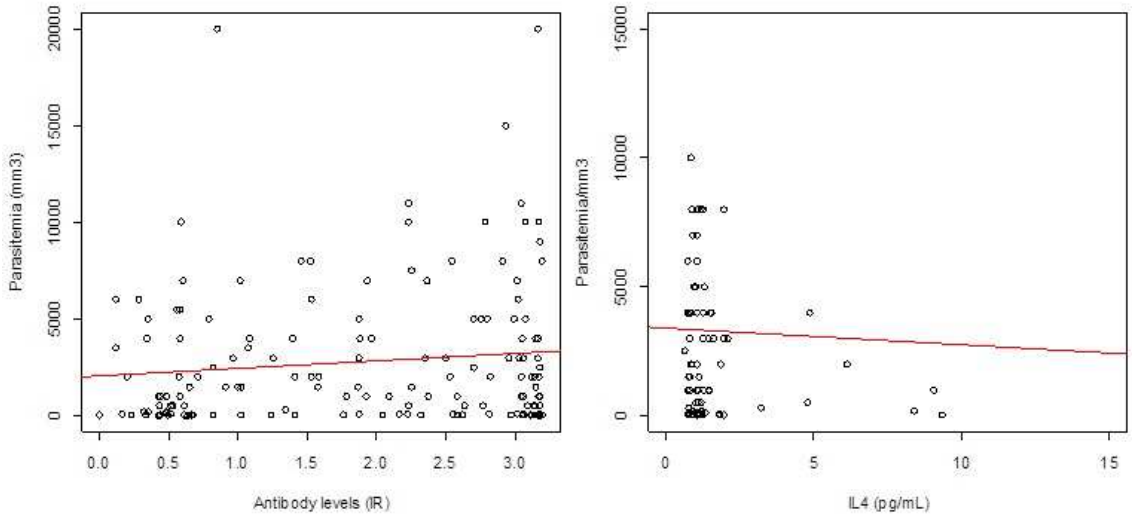


Figure 1. (a) Correlation between the levels of anti-AMA-1 antibodies and parasitemia. (b) Correlation between the IL-4 levels and parasitemia in 83 patients with *P. vivax* malaria

No significant differences were observed between the groups when compared according to gender, age, number of previous malaria episodes, exposure period and genetic ancestry (Table 1).

Table 1. Characteristics of malaria patients according to AMA-1 antibody response.

Characteristics	**All malaria patients** (n = 182)	**AMA-1 positive** (n = 124)	**AMA-1 negative** (n = 58)	**p-value**
Gender (male)[a]	69.8	71.7	65.5	0.49
Age (years)[b]	29 (14-68)	30 (14-68)	26 (14-65)	0.31
Exposure period (years)[b]	5 (0.1-63)	5 (0.1-37)	9 (0.1-63)	0.11
Previous malaria episodes[c]	4.7 ± 3.3	5.1 ± 3.1	3.8 ± 3.5	0.22
Primo-infected[a]	11.4	4.1	30.4	<0.0001
Genetic ancestry[c]				
African	0.320 ± 0.113	0.318 ± 0.117	0.326 ± 0.105	0.55
European	0.441 ± 0.121	0.441 ± 0.120	0.441 ± 0.123	0.90
Native Amerindian	0.238 ± 0.088	0.241 ± 0.092	0.233 ± 0.079	0.78
Parasite density (parasites/µL)[b]	1500 (5-10000)	2000 (10-10000)	1000 (5-7000)	0.09
IL-4 concentration (pg/mL)[b]	1.05 (0.615-40.365)	1.04 (0.725-9.325)	1.18 (0.615-40.365)	0.26

[a]Percentages

[b]Median (range)

[c]Mean ± standard deviation

As described in Table 2, for the polymorphism at position -1577 G/A, the G/A genotype was the most frequent (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 was the most common (86.7%) followed by the T/C genotype (12.3%), and the C/C genotype was the least frequent (1%).

Table 2. Genotype and allele frequencies of *CTLA4* gene polymorphisms in patients from the Brazilian Amazon region with vivax malaria.

Gene	**SNPs**	**Position in the gene**	**Genotype/ Allele**	**Frequency (%)**	**HWE**
CTLA4	rs733618	-1722	TT	163 (86.7%)	p=0.39
			TC	23 (12.3%)	
			CC	2 (1%)	
			T	349 (92.8%)	
			C	27 (7.2%)	
	rs11571316	-1577	GG	73 (41%)	p=0.24
			GA	88 (49.4%)	
			AA	17 (9.6%)	
			G	234 (65.7%)	
			A	122 (34.3%)	

No significant association was observed between genotypes evaluated at positions -1722 (p=0.68) and -1577 (p=0.74) for levels of IgG antibodies against PvAMA-1 (Figure 2a) or for IL-4 levels (Figure 2b). There was no significant difference between the genotype frequencies of responder and non-responder individuals for IgG against PvAMA-1 (Table 3).

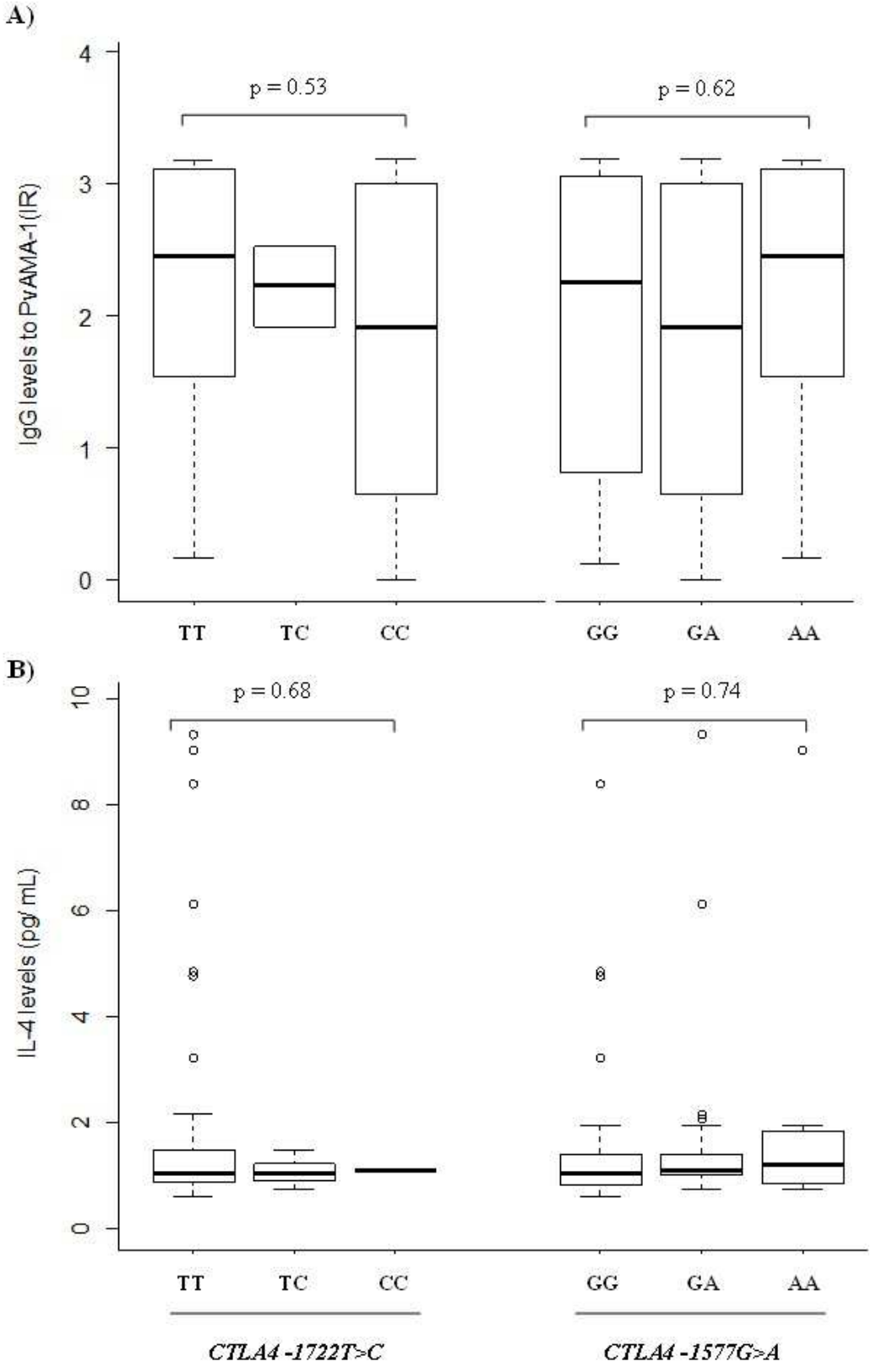


Figure 2. (a) Correlation between the levels of anti-AMA-1 antibodies and the genotypes at positions -1722 (T/C) and -1577 (G/A). (b) Correlation between the IL-4

levels and the genotypes at positions -1722 (T/C) and -1577 (G/A) in 83 patients with *P. vivax* malaria from the Brazilian Amazon region.

Table 3. Distribution of *CTLA4* polymorphisms among AMA-1 responding and non-responding malaria patients.

***CTLA4* Polymorphism**	**Frequency (%)**		**p-value**
	AMA-1 Positive	**AMA-1 Negative**	
-1722 T>C			0.58
TT	87.7	86.5	
TC	10.7	13.5	
CC	1.6	0	
-1577 G>A			0.55
GG	42.7	38.0	
GA	47.8	56.0	
AA	9.6	6.0	

No significant association was found between parasitemia and genotypes, with p=0.86 for the SNP at position -1722 and p=0.13 for the SNP at position -1577.

4. Discussion

The immune response against malaria is complex and involves innate and adaptive mechanisms that are able to eliminate the infectious agent or cause immunopathology (Medina et al., 2011). Immunogenic molecules present on the surface of the parasite have been widely studied as potential vaccine candidates, including circumsporozoite protein (CSP) (Herrera et al., 2005.), merozoite surface protein (MSP)

(Oliveira-Ferreira et al., 2004; Stowers et al., 2001; Valderrama-Aguirre et al., 2005) and Pv25 (Hisaeda et al., 2000; Malkin et al., 2005), and the proteins involved in the erythrocyte invasion process, such as duffy-binding protein (DBP) (Arévalo-Herrera et al., 2005; Ceravólo et al., 2005; Yazdani et al., 2004) and AMA-1 (Saul et al., 2005). Additionally, associations between molecules involved in the host's immune system and the susceptibility to acquiring infection by *P. vivax* in different endemic areas of the Brazilian Amazon have been described (Oliveira-Ferreira et al., 2004; Storti-Melo et al., 2012). However, the costimulatory molecules of the immune response have been little investigated. Our results comprise the first Brazilian study evaluating immune response mechanisms that correlates *CTLA4* gene polymorphisms and different biological aspects involved in *P. vivax* infection in an endemic area of the Brazilian Amazon.

The low IL-4 levels observed in the present study did not correlate with levels of parasitemia. Similar data were found in individuals infected with *P. vivax* in other endemic areas of the state of Pará (Medina et al., 2011). In the states of Acre and Amazonas, IL-4 levels were higher in asymptomatic individuals with low parasitemia from *P. vivax* (Gonçalves et al., 2012). Conflicting results were also observed in studies in Turkey (Zeyrek et al., 2006) with vivax malaria and in Thailand (Tangteerawatana et al., 2009) with *P. falciparum* malaria. An explanation for the differences in cytokine levels may be that these two *Plasmodium* species have distinct biological characteristics. In the study by Gonçalves et al. conducted in 2012, the negative correlation between high IL-4 levels and low parasitemia may indicate that this cytokine is involved in parasitemia control during the course of the disease. Another possible explanation for this result is that the disease in those individuals may have been in its early stage, when the parasitemia levels are still low. Moreover, IL-4 levels may vary

during different stages of infection (Medina et al., 2011). Thus for a better understanding of this mechanism, a longitudinal study would be required to more precisely determine the correlation between parasite burden and IL-4 levels at different stages of infection.

A study with cultures of splenic isolates from BALB/c mice reported that cross-linking *CTLA4* with *CD28* and the *CD3-TCR* complex led to inhibition of T cell proliferation and IL-2 secretion, as well as suppression of interferon-γ (Th1) and IL-4 (Th2) cells (Chen et al., 1998). The *CTLA4* gene is known to be expressed on the surface of activated T cells, but it is also expressed constitutively on the surface of Treg cells. Treg cells are important for the maintenance of immune homeostasis as they control excessive immune responses, but they may also promote the survival of pathogens. Higher frequencies of Treg cells have been associated with increased parasitemia in patients infected with *P. falciparum* (Minigo et al., 2009; Scholzen et al., 2009). Studies in animal models and *in vitro* studies have demonstrated that antibodies against AMA-1 may reduce parasite replication and protect against lethal infections (Remarque et al., 2008). In a study performed with monoclonal antibodies against *P. knowlesi* AMA-1, these antibodies were able to inhibit merozoite invasion *in vitro* (Thomas et al., 1984). Our results do not show association of IL-4 levels and antibodies against AMA-1 with *CTLA4* gene polymorphisms. Thus, stimulation of cytokine production and production of malaria antibodies in humans were not influenced by these polymorphisms. However, the role of CTLA-4 in IL-4 regulation is not yet well characterized. Studies have demonstrated that other molecules involved in the immune response may be involved in this process (Riley et al., 2001). Interestingly, several studies have shown that genetic SNPs, especially in the promoter region of the *CTLA4*

gene, are associated with infectious diseases (Idris et al, 2011; 2012), autoimmune diseases (Almasi et al., 2006; Taha Khalaf et al., 2011) and cancer (Su et al., 2007). Our results show that for the -1722 SNP, the homozygous TT genotype was predominant, with a frequency of 86.7%. In Gabon, this genotype has been associated with schistosomiasis (Idris et al., 2012), and in Iran, it has been associated with systemic sclerosis (Almasi et al., 2006). The prevalence of the heterozygous TC genotype in a sample of the Chinese population was associated with systemic lupus erythematosus (Taha Khalaf et al., 2011) and in a sample of the Korean population with Behçet's disease (Park et al., 2009). For the -1577 SNP, the heterozygous GA genotype had the highest frequency of 49.4%, and there was a predominance of the G allele with 65.7% frequency. A case-control study in Malaysia with lymphatic filariasis showed a predominance of the GA genotype (Idris et al., 2011).

Despite showing associations with other diseases, these *CTLA4* gene polymorphisms in vivax malaria do not seem to be an important factor for immune response regulation, as they do not influence the humoral immune response and consequently the development of parasitemia. These differences may result from several factors, such as genetic variability of the parasite, polymorphisms in other molecules involved in the immune response and the ethnic composition of the population of Goianésia do Pará because the Brazilian population is genetically diverse and is composed of three ethnic groups, Caucasians, Africans and Amerindians, which differ between regions (Pena et al., 2011; Sortica et al., 2012). Despite having no effects in the population studied, the *CTLA4* gene SNPs may be associated with vivax malaria in other endemic areas; thus, it is important to study the *CTLA4* gene polymorphisms in different areas and different ethnic groups.

Acknowledgements

We are grateful to all of the individuals who participated in this study. We would like to thank Irene da Silva Soares and Kátia Sanches Françoso for their assistance in the AMA-1 serological analysis.

References

Almasi, S., Erfani, N., Mojtahedi, Z., Rajaee, A., Ghaderi, A., 2006. Association of CTLA-4 gene promoter polymorphisms with systemic sclerosis in Iranian population. Genes Immun. 7, 401–406.

Arévalo-Herrera, M., Chitnis, C., Herrera, S., 2010. Current status of *Plasmodium vivax* vaccine. Hum. Vaccin. 6, 124–132.

Arévalo-Herrera, M., Castellanos, A., Yazdani, S.S., Shakri, A.R., Chitnis, C.E., Dominik, R., Herrera, S., 2005. Immunogenicity and protective efficacy of recombinant vaccine based on the receptor-binding domain of the *Plasmodium vivax* Duffy binding protein in Aotus monkeys. Am. J. Trop. Med. Hyg. 73, 25–31.

Bueno, L.L., Lobo, F.P., Morais, C.G., Mourão, L.C., de Ávila, R.A., Soares, I.S., Fontes, C.J., Lacerda, M.V., Chaves Olórtegui, C., Bartholomeu, D.C., Fujiwara, R.T., Braga, E.M., 2011. Identification of a highly antigenic linear B cell epitope within *Plasmodium vivax* apical membrane antigen 1 (AMA-1). PLoS ONE 6:e21289.

Ceravólo, I.P., Bruña-Romero, O., Braga, E.M., Fontes, C.J., Brito, C.F., Souza, J.M., Krettli A.U., Adams, J.H., Carvalho, L.H., 2005. Anti-*Plasmodium vivax* duffy binding protein antibodies measure exposure to malaria in the Brazilian Amazon. Am. J. Trop. Med. Hyg. 72, 675–681.

Chen, W., Jin, W., Wahl, S.M., 1998. Engagement of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) induces transforming growth factor beta (TGF-beta) production by murine CD4(+) T cells. J. Exp. Med. 188, 1849–1857.

Cheng, T.Y., Lin, J.T., Chen, L.T., Shun, C.T., Wang, H.P., Lin, M.T., Wang, T.E., Cheng, A.L., Wu, M.S., 2006. Association of T-cell regulatory gene polymorphisms with susceptibility to gastric mucosa-associated lymphoid tissue lymphoma. J. Clin. Oncol. 24, 3483–3489.

Elias, R.M., Sardinha, L.R., Bastos, K.R., Zago, C.A., Silva, A.P., Alvarez, J.M., Lima, M.R., 2005. Role of CD28 in polyclonal and specific T and B cell responses required for protection against blood stage malaria. J. Immunol. 174, 790–799.

Gonçalves, R.M., Scopel, K.K., Bastos, M.S., Ferreira, M.U., 2012. Cytokine balance in human malaria: does Plasmodium vivax elicit more inflammatory responses than Plasmodium falciparum? PLoS One. 7, e44394.

Herrera, S., Bonelo, A., Perlaza, B.L., Fernández, O.L., Victoria, L., Lenis, A.M., Soto, L., Hurtado, H., Acuña, L.M., Vélez, J.D., Palacios, R., Chen-Mok, M., Corradin, G., Arévalo-Herrera, M., 2005. Safety and elicitation of humoral and cellular responses in colombian malaria-naïve volunteers by a *Plasmodium vivax* circumsporozoite protein-derived synthetic vaccine. Am. J. Trop. Med. Hyg. 73, 3–9.

Hisaeda, H., Stowers, A.W., Tsuboi, T., Collins, W.E., Sattabongkot, J.S., Suwanabun, N., Torii, M. Kaslow, D.C., 2000. Antibodies to malaria vaccine candidates Pvs25 and Pvs28 completely block the ability of *Plasmodium vivax* to infect mosquitoes. Infect. Immun. 68, 6618–6623.

Idris, Z.M., Miswan, N., Muhi, J., Mohd, T.A., Kun, J.F., Noordin, R., 2011. Association of CTLA4 gene polymorphisms with lymphatic filariasis in an East Malaysian population. Hum. Immunol. 72, 607–612.

Idris, Z.M., Yazdanbakhsh, M., Adegnika, A.A., Lell, B., Issifou, S., Noordin, R., 2012. A pilot study on cytotoxic T lymphocyte-4 gene polymorphisms in urinary schistosomiasis. Genet. Test Mol. Biomarkers. 16, 488–492.

Jacobs, T., Graefe, S.E., Niknafs, S., Gaworski, I., Fleischer, B., 2002. Murine malaria is exacerbated by CTLA-4 Blockade. J. Immunol. 169, 2323–2329.

Jha, A.N., Singh, V.K., Kumari, N., Singh, A., Antony, J., van Tong, H., Singh, S., Pati, S.S., Patra, P.K., Singh, R., Toan, N.L., Song Ie, H., Assaf, A., Messias-Reason, I.J., Velavan, T.P., Singh, L., Thangaraj, K., 2012. IL-4 haplotype -590T, -34T and intron-3 VNTR R2 is associated with reduced malaria risk among ancestral indian tribal populations. PLoS One 7, e48136.

Liu, J., Zhang, H., 2013. -1722T/C polymorphism (rs733618) of CTLA-4 significantly associated with lupus erythematosus (SLE): a comprehensive meta-analysis. Hum. Immunol. 74, 341–347.

Malkin, E.M., Durbin, A.P., Diemert, D.J., Sattabongkot, J., Wu, Y., Miura, K., Long, C.A., Lambert, L., Miles, A.P., Wang, J., Stowers, A., Miller, L.H. Saul, A., 2005. Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for *Plasmodium vivax* malaria. Vaccine 23, 3131–3138.

Martins, G.A., Takodoro, C.E., Silva, R.B., Silva, J.S., Rizzo, L.V., 2004. CTLA-4 blockage increases resistance to infection with the intracellular protozoan *Trypanosoma cruzi*. J. Immunol. 172, 4893–4901.

Masteller, E.L., Chuang, E., Mullen, A.C., Reiner, S.L., Thompson, C.B., 2000. Structural analysis of CTLA-4 function in vivo. J. Immunol. 164, 5319–5327.

McCoy, K., Camberis, M., Gros, G.L., 1997. Protective immunity to nematode infection is induced by CTLA-4 blockade. J. Exp. Med. 186, 183–187.

Medina, T.S., Costa, S.P., Oliveira, M.D., Ventura, A.M.., Souza, J.M., Gomes, T.F., Vallinoto, A.C., Póvoa, M.M., Silva, J.S., Cunha, M.G., 2011. Increased interleukin-10 and interferon-g levels in *Plasmodium vivax* malaria suggest a reciprocal regulation which is not altered by IL-10 gene promoter polymorphism. Malar. J. 10, 264.

Minigo, G., Woodberry, T., Piera, K.A., Salwati, E., Tjitra, E., Kenangalem, E., Price, R.N., Engwerda, C.R., Anstey, N.M., Plebanski, M., 2009. Parasite-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria. PLoS Pathog. 5, e1000402.

Murphy, M.L., Cotterell, S.E., Gorak, P.M., Engwerda, C.R., Kaye, P.M., 1998. Blockade of CTLA-4 enhances host resistance to the intracellular pathogen *Leishmania donovani*. J. Immunol. 161, 4153–4160.

Oliveira-Ferreira, J., Lacerda, M.V., Brasil, P., Ladislau, J.L., Tauil, P.L., Daniel-Ribeiro, C.T., 2010. Malaria in Brazil: an overview. Malar. J. 9, 115.

Oliveira-Ferreira, J., Vargas-Serrato, E., Barnwell, J.W., Moreno, A., Galinski, M.R., 2004. Immunogenicity of *Plasmodium vivax* merozoite surface protein-9 recombinant proteins expressed in *E. coli*. Vaccine 22, 2023–2030.

Park, K.S., Baek, J.A., Do, J.E., Bang, D., Lee, E.S., 2009. CTLA4 gene polymorphisms and soluble CTLA4 protein in Behcet's disease. Tissue Antigen. 74, 222–227.

Pena, S.D., Di Pietro, G., Fuchshuber-Moraes, M., Genro, J.P., Hutz, M.H., Kehdy Fde, S., Kohlrausch, F., Magno, L.A., Montenegro, R.C., Moraes, M.O., de Moraes, M.E., de Moraes, M.R., Ojopi, E.B., Perini, J.A., Racciopi, C., Ribeiro-Dos-Santos, A.K., Rios-Santos, F., Romano-Silva, M.A., Sortica, V.A., Suarez-Kurtz, G., 2011. The genomic ancestry of individuals from different geographical regions of Brazil is more uniform than expected. PLoS One 6:e17063.

Pincerati, M.R., Dalla-Costa, R., Pavoni, D.P., Petzl-Erler, M.L., 2010. Genetic polymorphisms of the T-cell coreceptors CD28 and CTLA4 in Afro- and Euro-Brazilians. Int. J. Immunogenet. 37, 253–261.

Remarque, E.J., Faber, B.W., Kocken, C.H., Thomas, A.W., 2008. Apical membrane antigen 1: a malaria vaccine candidate in review. Trends Parasitol. 24, 74–84.
Riley, J.L., Blair, P.J., Musser, J.T., Abe, R., Tezuka, K., Tsuji, T., June, C.H., 2001. ICOS costimulation requires IL-2 and can be prevented by CTLA-4 engagement. J. Immunol. 15, 4943–4948.

Rudd, C.E., Schneider, H., 2003. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signaling. Nat. Rev. Immunol. 3, 544-556.

Rummel, T., Batchelder, J., Flaherty, P., LaFleur, G., Nanavati, P., Burns J.M., Weidanz, W.P., 2004. CD28 costimulation is required for the expression of T-cell-dependent cell-mediated immunity against blood-stage *Plasmodium chabaudi* malaria parasites. Infect. Immun. 72, 5768–5774.

Saul, A., Lawrence, G., Allworth, A., Elliott, S., Anderson, K., Rzepczyk, C., Martin, L.B., Taylor, D., Elisen, D.P., Irving, D.O., Pye, D., Crewther, P.E., Hodder, A.N., Murphy, V.J., Anders, R.F., 2005. A human phase 1 vaccine clinical trial of the Plasmodium falciparum malaria vaccine candidate apical membrane antigen 1 in Montanide ISA720 adjuvant. Vaccine 23, 3076–3083.

Sayegh, M.H., 1999. Finally, CTLA-4Ig graduates to the clinic. J. Clin. Invest. 103, 1223–1225.

Scholzen, A., Mittag, D., Rogerson, S.J., Cooke, B.M., Plebanski, M., 2009. *Plasmodium falciparum*-mediated induction of human CD25Foxp3 CD4 T cells is independent of direct TCR stimulation and requires IL-2, IL-10 and TGFbeta. PLoS Pathog. 5, e1000543.

Sistema de Informações de Vigilância Epidemiológica da Malária [Malaria Epidemiological Surveillance Information System] (SIVEP-Malária), n.d. http://portalweb04.saude.gov.br/sivep_malaria. Accessed May 2014.

Snounou, G., Viriyakosol, S., Jarra, W., Thaithong, S., Brown, K.N., 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infection. Mol. Biochem. Parasitol. 58, 283–292.

Sortica, V.A., Cunha, M.G., Ohnishi, M.D., Souza, J.M., Ribeiro-Dos-Santos, A.K., Santos, N.P., Callegari-Jacques, S.M., Santos, S.E., Hutz, M.H., 2012. IL1B, IL4R, IL12RB1 and TNF gene polymorphisms are associated with *Plasmodium vivax* malaria in Brazil. Malar. J. 11, 409. doi: 10.1186/1475-2875-11-409.

Storti-melo, L.M., da Costa, D.R., Souza-Neiras, W.C., Cassiano, G.C., Couto, V.S., Póvoa, M.M., Soares Ida, S., de Carvalho, L.H., Arevalo-Herrera, M., Herrera, S., Rossit, A.R., Cordeiro, J.A., de Mattos, L.C., Machado, R.L., 2012. Influence of HLA-DRB-1 alleles on the production of antibody against CSP, MSP-1, AMA-1, and DBP in Brazilian individuals naturally infected with Plasmodium vivax. Acta Tropica. 121, 152–155.

Stowers, A.W., Cioce, V., Shimp, R.L., Lawson, M., Hui, G., Muratova, O., Kaslow, D.C., Robinson, R., Long, C.A., Miller, L.H., 2001. Efficacy of two alternate vaccines based on *Plasmodium falciparum* merozoite surface protein 1 in an Aotus challenge trial. Infect. Immun. 69, 1536–1546.

Su, T.H., Chang, T.Y., Lee, Y.J., Chen, C.K., Liu, H.F., Chu, C.C., Lin, M., Wang, P.T., Huang, W.C., Chen, T.C., Yang, Y.C., 2007. CTLA-4 gene and susceptibility to human papillomavirus-16-associated cervical squamous cell carcinoma in Taiwanese woman. Carcinogenesis 28, 1237–1240.

Taha Khalaf, A., Song, J.Q., Gao, T.T., Yu, X.P., Lei, T.C., 2011. CTLA-4 gene polymorphism and the risk of systemic lupus erythematosus in the Chinese population. J. Biomed. Biotechnol. 2011, 167395.

Tangpukdee, N., Duangdee, C., Wilairatana, P., Krudsood, S., 2009. Malaria Diagnosis: A Brief Review. Korean J. Parasitol. 47, 93-102.

Tangteerawatana, P., Perlmann, H., Hayano, M., Kalambaheti, T., Troye-Blomberg, M., Khusmith, S., 2009. IL4 gene polymorphism and previous malaria experiences manipulate anti-*Plasmodium falciparum* antibody isotype profiles in complicated and uncomplicated malaria. Malar. J. 8, 286. doi: 10.1186/1475-2875-8-286.

Thomas, A.W., Deans, J.A., Mitchell, G.H., Alderson, T., Cohen, S., 1984. The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit Plasmodium knowlesi invasion of erythrocytes. Mol. Biochem. Parasitol. 13, 187–199.

Valderrama-Aguirre, A., Quintero, G., Gómez, A., Castellanos, A., Pérez, Y., Méndez, F., Arévalo-Herrera, M., Herrera, S., 2005. Antigenicity, immunogenicity and protective efficacy of *Plasmodium vivax* MSP1 PV200l: a potential malaria vaccine subunit. Am. J. Trop. Med. Hyg. 73, 16–24.

Vicentin, E.C., Françoso, K.S., Rocha, M.V., Iourtov, D., Dos Santos, F.L., Kubrusly, F.S., Sakauchi, M.A., Raw, I., Nosten, F., Rénia, L., Rodrigues, M.M., Russell, B., Soares, I.S., 2014. Invasion-inhibitory antibodies elicited by immunization with *Plasmodium vivax* apical membrane antigen-1 expressed in Pichia pastoris yeast. Infect. Immun. 82, 1296–1307. doi:10.1128/IAI.01169-13

Warnes, G., Gorjanc, G., Leisch, F., Man, M., 2011. Genetics: Population Genetics. R package version 1.3.6. http://CRAN.R-project.org/package=genetics. Accessed 30 July 2013.

World Health Organization (WHO). World malaria report 2013. Geneva (Switzerland): World Health Organization; 2013.

Yazdani, S.S., Shakri, A.R., Mukherjee, P., Baniwal, S.K., Chitnis, C.E., 2004. Evaluation of immune responses elicited in mice against a recombinant malaria vaccine based on *Plasmodium vivax* Duffy binding protein. Vaccine 22, 3727–3737.

Zakeri, S., Sadeghi, H., Mehrizi, A.A., Djadid, N.D., 2013. Population genetic structure and polymorphism analysis of gene encoding apical membrane antigen-1 (AMA-1) of Iranian *Plasmodium vivax* wild isolates. Acta Tropica. 126, 269–279.

Zeyrek, F.Y., Kurcer, M.A., Zeyrek, D., Simsek, Z., 2006. Parasite density and serum cytokine levels in P*lasmodium vivax* malaria in Turkey. Parasite Immunol. 28, 201–207

ARTIGO 2

Artigo 2

>From: ehi@ashi-hla.org
>To: gcapatti@hotmail.com
>Date: Mon, 21 Apr 2014 16:13:06 +0100
>Subject: A manuscript number has been assigned
>
>Ms. Ref. No.: HIM-D-14-00166
>Title: Impact of population admixture on the distribution of immune response co-stimulatory genes polymorphisms in a Brazilian population
>Human Immunology
>
>Dear Dr. Gustavo Capatti Cassiano,
>
>Your submission entitled "Impact of population admixture on the distribution of immune response co-stimulatory genes polymorphisms in a Brazilian population" has been been assigned the following manuscript number: HIM-D-14-00166.
>
>You may check on the progress of your paper by logging on to the Elsevier Editorial System as an author. The URL is http://ees.elsevier.com/him/.
>
>Your username is: gcapatti@hotmail.com
>
>If you need to retrieve password details, please go to:
>
>http://ees.elsevier.com/him/automail_query.asp
>
>Thank you for submitting your work to this journal.
>
>Kind regards,

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

Gustavo Capatti Cassiano[a,b,*], Eduardo José Melo Santos[c], Maria Helena Thomaz Maia[c], Adriana da Cruz Furini[b], Luciane Moreno Storti-Melo[d], Franciele Maira Batista Tomaz[b], Pamella Cristina Alves Trindade[b], Marcela Petrolini Capobianco[a,b], Marcos Antônio Trindade Amador[e], Giselle Maria Rachid Viana[f], Marinete Marins Póvoa[f], Sidney Emanuel Batista Santos[e], Ricardo Luiz Dantas Machado[a,f]

[a]Universidade Estadual Paulista, Instituto de Biociências, Letras e Ciências Exatas, São José do Rio Preto, Brazil

[b]Faculdade de Medicina de São José do Rio Preto, Centro de Investigação de Microrganismos, São José do Rio Preto, Brazil

[c]Universidade Federal do Pará, Instituto de Ciências Biológicas, Belém, Brazil

[d]Universidade Federal do Sergipe, Departamento de Biociências, Itabaiana, Brazil

[e]Universidade Federal do Pará, Laboratório de Genética Humana e Médica, Belém, Brazil

[f]Laboratório de Pesquisas Básicas em Malária, Instituto Evandro Chagas, Secretaria de Vigilância em Saúde, Ananindeua, Brazil

*Corresponding Author

Gustavo Capatti Cassiano

Departamento de Doenças Dermatológicas, Infeciosas e Parasitárias

Faculdade de Medicina de São José do Rio Preto

Avenida Brigadeiro Faria Lima, 5416

São José do Rio Preto, Brazil

Email: gcapatti@hotmail.com

Telephone/fax number: +55 17 32015736

Abbreviated Title: Population structure and co-stimulatory genes

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* e *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.

Keywords: admixture population; ancestry markers; immunogenetics

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 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 investigated associations between autoimmune diseases and the 2q33 region, mainly focusing on *CTLA4* gene polymorphisms [3]. Two *single nucleotide polymorphisms (*SNPs) in this gene, one located in the promoter region (rs5742909) and the other located in exon 1 (rs231775) have been associated with various diseases. The latter has previously been shown to have a functional effect on the expression of CTLA-4 [4]. The diseases associated with these two SNPs in different ethnic groups include type 1 diabetes [5], Graves' disease [6], rheumatoid arthritis [7], pemphigus foliaceus [8], systemic sclerosis [9], and systemic lupus erythematosus [10]. Little is known about the functional role of other polymorphisms in the 2q33 region. One SNP, rs3116496, in the third intron of the *CD28* gene, is located close to a splicing site and so it is suggested that this polymorphism may affect the expression of the molecule; it has been associated with cervical cancer [11] and rheumatoid arthritis [12]. Teutsch et al. [13] detected another polymorphism (rs35593994) a substitution of guanine by adenine in the promoter region of the *CD28* gene in Australians. These authors suggested possible implications of this SNP on gene expression but it has not been investigated in other populations with the exception of one study in Brazil [14]. Another SNP which has been poorly investigated is the rs4404254 polymorphism in the *ICOS* gene; allele frequencies are only available for Europeans (in particular Scandinavians) [15] and Brazilians [16,

17]. The rs1129055 polymorphism in the *CD86* gene has been extensively studied in Asians. This SNP, located in exon 8, causes a substitution of alanine for threonine and has been associated with asthma [18] and osteosarcoma [19].

Recently, after recognizing that B cells as well as T cells may be important in the development of autoimmune diseases, polymorphisms in genes that participate in the co-stimulation of these cells have started to be investigated. Of these, the most studied polymorphism, the rs1883832 SNP in the *CD40* gene, has been investigated in some diseases, including multiple sclerosis [20], non-Hodgkin lymphoma [21], and osteoporosis [22]. The rs3092945 polymorphism in the *CD40L* gene located on X chromosome has been studied almost exclusively in African populations due to its association with the most severe forms of malaria falciparum [23, 24]. In respect to the *BLYS* gene, the rs9514828 polymorphism has been associated with systemic lupus erythematosus [25] and idiopathic thrombocytopenic purpura [26].

Although several studies have shown associations between polymorphisms in co-stimulatory genes and diseases, studies in other populations have failed to reproduce the results. 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 [27]. 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 [28,

29]. 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 [30, 31]. 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^{TM} 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 1x Buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 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 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at an annealing temperature depending on the primer and 1 minute at 72°C, and a final step of 10 minutes 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 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 [32]. The markers were selected employing two main criteria: significant differences in allele frequencies

between Africans, Europeans and/or Amerindians (≥ 40%) and located on different chromosomes or in distant physical regions when on the same chromosome. 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. [32].

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 [33]. 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 [34]. 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 [35]. 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 Table 3.

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, 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).

The population was divided into four quartiles according to the proportion of ancestry (< 0.25; 0.25-0.50; 0.50-0.75; > 0.75) and the allele frequencies of all the SNPs were determined for each quartile. The Chi-square test for trend identified a significant decrease in the frequency of the *T* allele of the rs3092945 SNP in the *CD40L* gene parallel to the increase in African ancestry (p-value = 0.01) and a significant increase in the frequency of this allele together with increases in European ancestry (p-value = 0.007). In relation to the rs4404254 *T* allele of the *ICOS* gene, there was a significant

decrease in the frequency of this allele as African ancestry increased (p-value < 0.00001).

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 Figure 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 (Table 4).

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 5). 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.

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 Figure 2.

4. Discussion

Several studies on the genomic ancestry of the Brazilian population corroborate historical data of a tri-hybrid parental composition (Europeans, Africans and Amerindians) [28, 32, 39]. 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% [32, 40, 41]. 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 [28].

Nine polymorphisms in seven co-stimulatory genes (*CD28*, *CTLA4*, *ICOS*, *CD86*, *CD40*, *CD40L* and *BLYS*) were analyzed in this study; we evaluated the impact of the Brazilian population admixture on the distribution of these polymorphisms. We report a significant decrease in the frequency of the *T* allele for rs4404254 SNP in the *ICOS* gene with increasing African ancestry. The frequency of the *T* allele found in this study was lower than in two Northern Europe populations [42, 43]. This difference probably reflects the substantial African contribution in the sample of the current study. According to available data from 1000Genomes project, the *T* allele frequency is lower in populations of African origin.

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 data from 1000Genomes project, 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.

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 [44-46], but

information about the frequency of this SNP in other populations are scarce. Our results are in accordance with data available from 1000Genomes project, which report a higher frequency of the allele in the European population compared to an African population. Beltrame et al. [36] 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 (Table 3). Differences were only found with the rs3116496 and rs1883832 SNPs [16, 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 [28, 29]. 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. [14], 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. [47] 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. Previous studies on the haplotype diversity of other genes have shown differences in haplotype frequencies between the Brazilian population and other populations, including Europeans and Africans [48, 49].

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 co-stimulatory 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.

Acknowledgments

To David Andrew Hewitt for the English revision of this manuscript. Financial support was provided by National Counsel of Technological and Scientific Development (CNPq).

References

[1] Greenwald RJ, Freeman GJ, Sharpe AH. The B7 Family Revisited. Annu Rev Immunol 2005; 23:515-48.

[2] Scholz JL, Oropallo MA, Sindhava V, Goenka R, Cancro MP. The role of B lymphocyte stimulator in B cell biology: implications for the treatment of lupus. Lupus 2013; 22:350-60.

[3] Deitiker P, Atassi MZ. Non-MHC genes linked to autoimmune disease. Crit Rev Immunol 2012; 32:193-285.

[4] Mäurer M, Loserth S, Kolb-Mäurer A, Ponath A, Wiese S, Kruse N, Rieckmann P. A polymorphism in the human cytotoxic T-lymphocyte antigen 4 (CTLA-4) gene (exon 1 + 49) alters T-cell activation. Immunogenetics 2002; 54:1-8.

[5] Benmansour J, Stayoussef M, Al-Jenaidi FA, Rajab MH, Rayana CB, Said HB, Mahjoub T, Almawi WY. Association of single nucleotide polymorphisms in cytotoxic T-lymphocyte antigen 4 and susceptibility to autoimmune type 1 diabetes in Tunisians. Clin Vaccine Immunol 2010; 17:1473-7.

[6] Si X, Zhang X, Tang W, Luo Y. Association between the CTLA-4 +49A/G polymorphism and Graves' disease: A meta-analysis. Exp Ther Med 2012; 4:538-44.

[7] Li X, Zhang C, Zhang J, Zhang Y, Wu Z, Yang L, Xiang Z, Qi Z, Zhang X, Xiao X. Polymorphisms in the CTLA-4 gene and rheumatoid arthritis susceptibility: a meta-analysis. J Clin Immunol 2012; 32:530-9.

[8] Dalla-Costa R, Pincerati MR, Beltrame MH, Malheiros D, Petzl-Erler ML. Polymorphisms in the 2q33 and 3q21 chromosome regions including T-cell coreceptor and ligand genes may influence susceptibility to pemphigus foliaceus. Hum Immunol 2010; 71:809-17.

[9] Song GG, Lee YH. The CTLA-4 and MCP-1 Polymorphisms and susceptibility to systemic sclerosis: a meta-analysis. Immunol Invest 2013; 42:481-92.

[10] Hughes T, Adler A, Kelly JA, Kaufman KM, Williams AH, Langefeld CD, Brown EE, Alarcón GS, Kimberly RP, Edberg JC, Ramsey-Goldman R, Petri M, Boackle SA, Stevens AM, Reveille JD, Sanchez E, Martín J, Niewold TB, Vilá LM, Scofield RH, Gilkeson GS, Gaffney PM, Criswell LA, Moser KL, Merrill JT, Jacob CO, Tsao BP, James JA, Vyse TJ, Alarcón-Riquelme ME; BIOLUPUS Network, Harley JB, Richardson BC, Sawalha AH. Evidence for gene-gene epistatic interactions among susceptibility loci for systemic lupus erythematosus. Arthritis Rheum 2012; 64:485-92.

[11] Chen X, Li H, Qiao Y, Yu D, Guo H, Tan W, Lin D. Association of CD28 gene polymorphism with cervical cancer risk in a Chinese population. Int J Immunogenet 2011; 38:51-4.

[12] Ledezma-Lozano IY, Padilla-Martínez JJ, Leyva-Torres SD, Parra-Rojas I, Ramírez-Dueñas MG, Pereira-Suárez AL, Rangel-Villalobos H, Ruiz-Quezada SL, Sánchez-Hernández PE, Muñoz-Valle JF. Association of CD28 IVS3 +17T/C polymorphism with soluble CD28 in rheumatoid arthritis. Dis Markers 2011; 30:25-9.

[13] Teutsch SM, Booth DR, Bennetts BH, Heard RN, Stewart GJ. Association of common T cell activation gene polymorphisms with multiple sclerosis in Australian patients. J Neuroimmunol 2004; 148:218-30.

[14] Pincerati MR, Dalla-Costa R, Pavoni DP, Petzl-Erler ML. Genetic polymorphisms of the T-cell coreceptors CD28 and CTLA-4 in Afro- and Euro-Brazilians. Int J Immunogenet 2010; 37:253-61.

[15] Haimila KE, Partanen JA, Holopainen PM. Genetic polymorphism of the human ICOS gene. Immunogenetics 2002; 53:1028-32.

[16] Guzman VB, Morgun A, Shulzhenko N, Mine KL, Gonçalves-Primo A, Musatti CC, Gerbase-Delima M. Characterization of CD28, CTLA4, and ICOS polymorphisms in three Brazilian ethnic groups. Hum Immunol 2005; 66:773-6.

[17] Pendeloski KP, Sass N, Torloni MR, Mattar R, Moron AF, Franchim CS, Daher S. Immunoregulatory gene polymorphisms in women with preeclampsia. Hypertens Res 2011; 34:384-8.

[18] Corydon TJ, Haagerup A, Jensen TG, Binderup HG, Petersen MS, Kaltoft K, Vestbo J, Kruse TA, Børglum AD. A functional CD86 polymorphism associated with asthma and related allergic disorders. J Med Genet 2007; 44:509-15.

[19] Wang W, Song H, Liu J, Song B, Cao X. CD86 +1057G/A polymorphism and susceptibility to osteosarcoma. DNA Cell Biol 2011; 30:925-9.

[20] Sokolova EA, Malkova NA, Korobko DS, Rozhdestvenskii AS, Kakulya AV, Khanokh EV, Delov RA, Platonov FA, Popova TY, Aref' eva EG, Zagorskaya NN, Alifirova VM, Titova MA, Smagina IV, El' chaninova SA, Popovtseva AV, Puzyrev VP, Kulakova OG, Tsareva EY, Favorova OO, Shchur SG, Lashch NY, Popova NF, Popova EV, Gusev EI, Boyko AN, Aulchenko YS, Filipenko ML. Association of SNPs of CD40 gene with multiple sclerosis in Russians. PLoS One 2013; 8:e61032.

[21] Nieters A, Bracci PM, de Sanjosé S, Becker N, Maynadié M, Benavente Y, Foretova L, Cocco P, Staines A, Holly EA, Boffetta P, Brennan P, Skibola CF. A functional TNFRSF5 polymorphism and risk of non-Hodgkin lymphoma, a pooled analysis. Int J Cancer 2011; 128:1481-5.

[22] Pineda B, Tarín JJ, Hermenegildo C, Laporta P, Cano A, García-Pérez MÁ. Gene interaction between CD40 and CD40L reduces bone mineral density and increases osteoporosis risk in women. Osteoporos Int 2011; 221451-8.

[23] Sabeti P, Usen S, Farhadian S, Jallow M, Doherty T, Newport M, Pinder M, Ward R, Kwiatkowski D. CD40L association with protection from severe malaria. Genes Immun 2002; 3:286-91.

[24] Manjurano A, Clark TG, Nadjm B, Mtove G, Wangai H, Sepulveda N, Campino SG, Maxwell C, Olomi R, Rockett KR, Jeffreys A; MalariaGen Consortium, Riley EM, Reyburn H, Drakeley C. Candidate human genetic polymorphisms and severe malaria in a Tanzanian population. PLoS One 2012; 7:e47463.

[25] Zayed RA, Sheba HF, Abo Elazaem MA, Elsaadany ZA, Elmessery LO, Mahmoud JA, Abdel Rahman DR, Abdou FR. B-cell activating factor promoter polymorphisms in egyptian patients with systemic lupus erythematosus. Ann Clin Lab Sci 2013; 43:289-94.

[26] Emmerich F, Bal G, Barakat A, Milz J, Mühle C, Martinez-Gamboa L, Dörner T, Salama A. High-level serum B-cell activating factor and promoter polymorphisms in patients with idiopathic thrombocytopenic purpura. Br J Haematol 2007; 136:309-14.

[27] Holopainen P, Naluai AT, Moodie S, Percopo S, Coto I, Clot F, Ascher H, Sollid L, Ciclitira P, Greco L, Clerget-Darpoux F, Partanen J; Members of the European

Genetics Cluster on Coeliac Disease. Candidate gene region 2q33 in European families with coeliac disease. Tissue Antigens 2004; 63:212-22.

[28] Pena SD, Di Pietro G, Fuchshuber-Moraes M, Genro JP, Hutz MH, Kehdy Fde S, Kohlrausch F, Magno LA, Montenegro RC, Moraes MO, de Moraes ME, de Moraes MR, Ojopi EB, Perini JA, Racciopi C, Ribeiro-Dos-Santos AK, Rios-Santos F, Romano-Silva MA, Sortica VA, Suarez-Kurtz G. The genomic ancestry of individuals from different geographical regions of Brazil is more uniform than expected. PLoS One 2011; 6:e17063.

[29] Cardena MM, Ribeiro-Dos-Santos A, Santos S, Mansur AJ, Pereira AC, Fridman C. Assessment of the relationship between self-declared ethnicity, mitochondrial haplogroups and genomic ancestry in Brazilian individuals. PLoS One 2013; 8:e62005.

[30] Vargens DD, Almendra L, Struchiner CJ, Suarez-Kurtz G. Distribution of the GNB3 825C>T polymorphism among Brazilians: impact of population structure. Eur J Clin Pharmacol 2008; 64:253-6.

[31] Suarez-Kurtz G, Pena SD, Struchiner CJ, Hutz MH. Pharmacogenomic Diversity among Brazilians: Influence of Ancestry, Self-Reported Color, and Geographical Origin. Front Pharmacol 2012; 3:191.

[32] Santos NP, Ribeiro-Rodrigues EM, Ribeiro-Dos-Santos AK, Pereira R, Gusmão L, Amorim A, Guerreiro JF, Zago MA, Matte C, Hutz MH, Santos SE. Assessing

individual interethnic admixture and population substructure using a 48-insertion-deletion (INSEL) ancestry-informative marker (AIM) panel. Hum Mutat 2010; 31:184-90.

[33] Warnes G, Gorjanc G, Leisch F, Man M. 2011. Genetics: Population Genetics. R package version 1.3.6. http://CRAN.R-project.org/package=genetics. Accessed 30 July 2012.

[34] Sinnwell JP, Schaid DJ. 2009. haplo.stats: Statistical analysis of haplotypes with traits and covariates when linkage phase is ambiguous. R package version 1.4.4. http://CRAN.R-project.org/package=haplo.stats. Accessed 29 April 2013.

[35] Wickham H. ggplot2: Elegant Graphics for Data Analysis. Springer, New York; 2009.

[36] Beltrame MH, Pincerati MR, Dalla-Costa R, Wassem R, Köhler KF, Chautard-Freire-Maia EA, Tsuneto LT, Petzl-Erler ML. CD80 and CD86 polymorphisms in populations of various ancestries: 5 new CD80 promoter alleles. Hum Immunol 2012; 73:111-7.

[37] Malheiros D, Petzl-Erler ML. Individual and epistatic effects of genetic polymorphisms of B-cell co-stimulatory molecules on susceptibility to pemphigus foliaceus. Genes Immun 2009; 10:547-58.

[38] Capobianco MP, Cassiano GC, Furini AA, Storti-Melo LM, Pavarino EC, Galbiatti AL, Fraga VD, Conceição LM, Couto VS, Couto AA, Machado RL. No evidence for association of the CD40, CD40L and BLYS polymorphisms, B-cell co-stimulatory molecules, with Brazilian endemic *Plasmodium vivax* malaria. Trans R Soc Trop Med Hyg 2013; 107:377-83.

[39] Francez PA, Ribeiro-Rodrigues EM, dos Santos SE. Allelic frequencies and statistical data obtained from 48 AIM INDEL loci in an admixed population from the Brazilian Amazon. Forensic Sci Int Genet 2012; 6:132-5.

[40] Pedroza LS, Sauma MF, Vasconcelos JM, Takeshita LY, Ribeiro-Rodrigues EM, Sastre D, Barbosa CM, Chies JA, Veit TD, Lima CP, Oliveira LF, Henderson BL, Castro AP, Maia MH, Barbosa FB, Santos SE, Guerreiro JF, Sena L, Santos EJ. Systemic lupus erythematosus: association with KIR and SLC11A1 polymorphisms, ethnic predisposition and influence in clinical manifestations at onset revealed by ancestry genetic markers in an urban Brazilian population. Lupus 2011; 20:265-73.

[41] Tarazona-Santos E, Castilho L, Amaral DR, Costa DC, Furlani NG, Zuccherato LW, Machado M, Reid ME, Zalis MG, Rossit AR, Santos SE, Machado RL, Lustigman S. Population genetics of GYPB and association study between GYPB*S/s polymorphism and susceptibility to *P. falciparum* infection in the Brazilian Amazon. PLoS One 2011; 6:e16123.

[42] Haimila K, Smedberg T, Mustalahti K, Mäki M, Partanen J, Holopainen P. Genetic association of coeliac disease susceptibility to polymorphisms in the ICOS gene on chromosome 2q33. Genes Immun 2004; 5:85-92.

[43] Ivansson EL, Juko-Pecirep I, Gyllensten UB. Interaction of immunological genes on chromosome 2q33 and IFNG in susceptibility to cervical cancer. Gynecol Oncol 2010; 116:544-8.

[44] Liao WL, Chen RH, Lin HJ, Liu YH, Chen WC, Tsai Y, Wan L, Tsai FJ. The association between polymorphisms of B7 molecules (CD80 and CD86) and Graves' ophthalmopathy in a Taiwanese population. Ophthalmology 2011; 118:553-7.

[45] Xiang H, Zhao W, Sun Y, Qian W, Xing J, Zhou Y, Yao J, Xu J, Wang Y, Yao H, Hu Z. CD86 gene variants and susceptibility to pancreatic cancer. J Cancer Res Clin Oncol 2012; 138:2061-7.

[46] Liu CP, Jiang JA, Wang T, Liu XM, Gao L, Zhu RR, Shen Y, Wu M, Xu T, Zhang XG. CTLA-4 and CD86 genetic variants and haplotypes in patients with rheumatoid arthritis in southeastern China. Genet Mol Res 2013; 12:1373-82.

[47] Butty V, Roy M, Sabeti P, Besse W, Benoist C, Mathis D. Signatures of strong population differentiation shape extended haplotypes across the human CD28, CTLA4, and ICOS costimulatory genes. Proc Natl Acad Sci USA 2007; 104:570-5.

[48] Moraes MO, Santos AR, Schonkeren JJ, Vanderborght PR, Ottenhoff TH, Moraes ME, Moraes JR, Sampaio EP, Sarno EN, Huizinga TW. Interleukin-10 promoter haplotypes are differently distributed in the Brazilian versus the Dutch population. Immunogenetics 2003; 54:896-9

[49] Lins TC, Vieira RG, Grattapaglia D, Pereira RW. Population analysis of vitamin D receptor polymorphisms and the role of genetic ancestry in an admixed population. Genet Mol Biol 2011; 34:377-85.

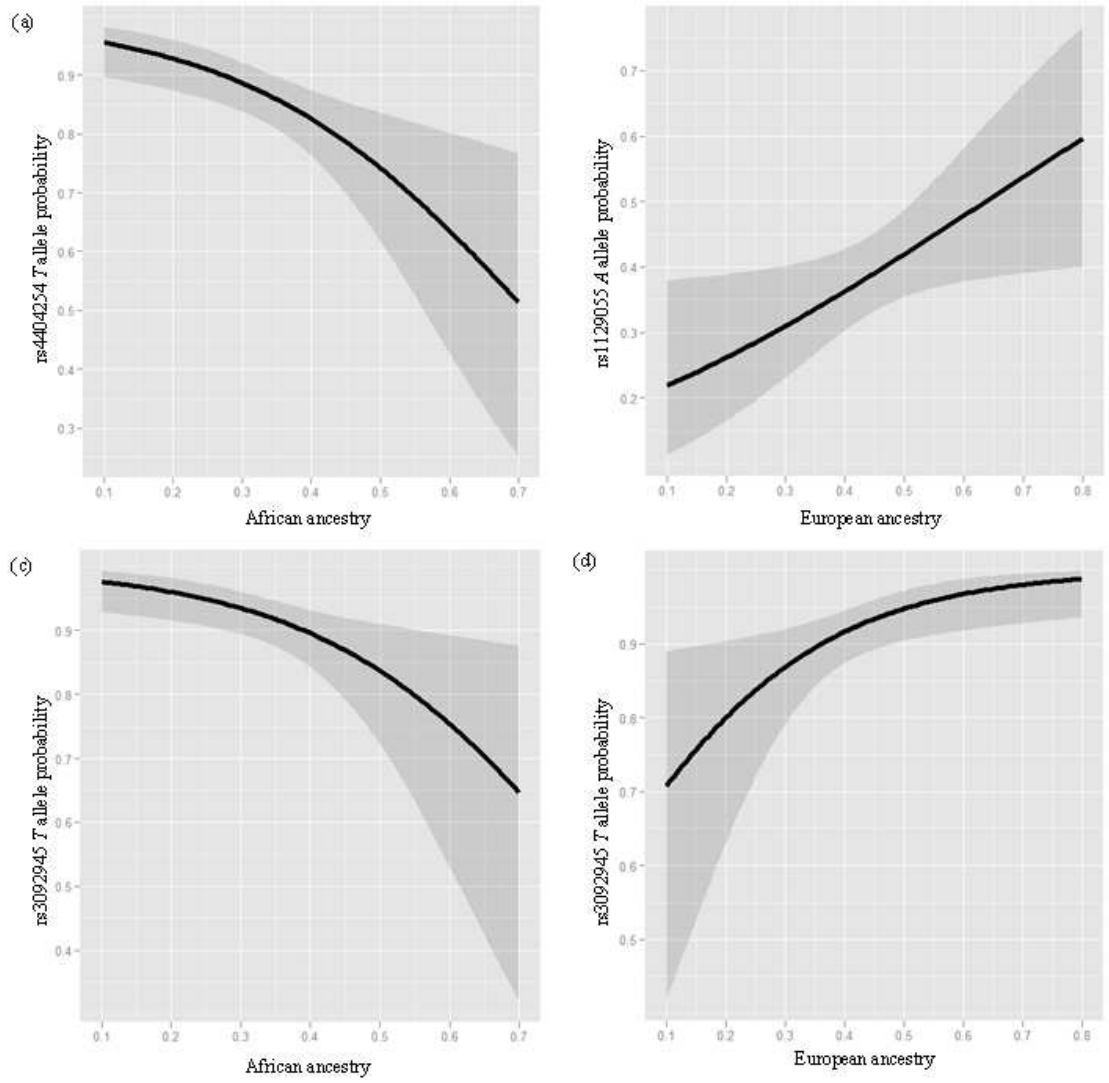


Fig. 1 Fitted logistic regression describing the association between ancestry and polymorphisms. (a) *ICOS* rs4404254. Chance of having a *T* allele according to African ancestry. (b) *CD86* rs1129055. Chance of having a *A* allele according to European ancestry. (c) *CD40L* rs3092945. Chance of having 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.

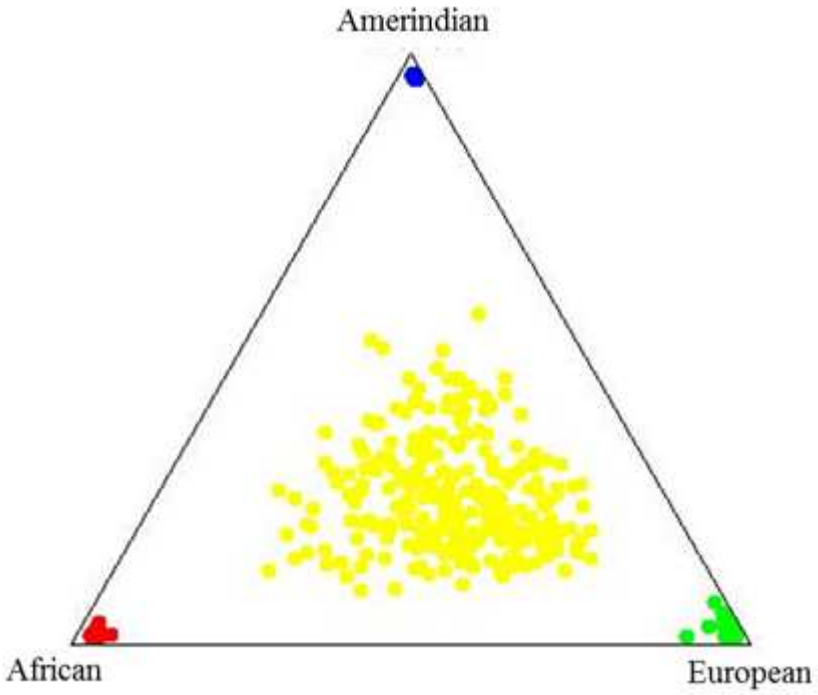


Fig. 2 Schematic representation of the individual admixture estimates. Each point represents one individual and the correspondent admixture proportions are indicated by the distance to the edges of the triangle. European, African and Amerindian correspond to individuals from the parental populations.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

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	*Hinf*I	G: 468 + 78 A: 546
rs3116496	*CD28* (2q33)	204594512	GAAACACCTTTGTCCAAGTC	CTCAATGCCTTCTGGGAAATC	52	*Aci*I	T: 333 C: 193 + 140
rs5742909	*CTLA4* (2q33)	204732347	GGGATTTAGGAGGACCCTTG	GTGCACACACAGAAGGCACT	48	*Mse*I	C: 244 T: 179 + 65
rs231775	*CTLA4* (2q33)	204732714	CTGAACACCGCTCCCATAAA	CACTGCCTTTGACTGCTGAA	50	*Bbv*I	A: 215 G: 159 + 56
rs4404254	*ICOS* (2q33)	204819570	TTACCAAGACTTTAGATGCTTTCTT	GAATCTTTCTAGCCAAATCATATTC	55	*Alu*I	T: 385 + 339 + 99 C: 339 + 289 + 99 + 96
rs1129055	*CD86* (3q21)	121838319	CTGTTCCAATGGCAACCTCT	GGTTGCCCAGGAACTTACAA	56	*Cvi*KI-1	G: 79 + 75 + 58 + 54 A: 154 + 58 + 54
rs3092945	*CD40L* (Xq26)	135729609	ATCTTCACAGCAACCTAC	CACTAAACTCAATGAAAGCC	56	*Lwe*I	T: 251 + 195 C: 446
rs1883832	*CD40* (20q12-q13.2)	44746982	GAAACTCCTGCGCGGTGAAT	GAAACTCCTGCGCGGTGAAT	56	*Sty*I	C: 133 + 96 + 74 T: 207 + 96
rs9514828	*BLYS* (13q32-q34)	108921373	TGGCTCTTGTGTGATCAAGG	GCCTGGTCTCAGCTTTTCTG	50	*Mbi*I	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	

[a]Ancestry expressed as mean (95% CI)

Table 3 Minor allele frequencies (MAF) in the samples from 1000Genomes populations and Brazilian populations of current and previous studies

SNP		BRZ (current study)	AFR	AMR	ASN	EUR	Euro-BRZ	Afro-BRZ	BRZ (mixed)	Reference
rs35593994	*CD28*	0,30	0,26	0,35	0,28	0,38	0,37[a]	0,33[a]	NA	(14)
rs3116496	*CD28*	0,19	0,04	0,13	0,09	0,22	0,19[a] 0,27[b]	0,15[a] 0,14[b]	0,15 0,16	(14, 16, 17)
rs5742909	*CTLA4*	0,08	0,01	0,08	0,11	0,09	0,10[a] 0,08[b]	0,09[a] 0,06[b]	0,11	(14, 16)
rs231775	*CTLA4*	0,34	0,40	0,41	0,64	0,36	0,34[a] 0,30[b]	0,37[a] 0,35[b]	0,27 0,32	(14, 16, 17)
rs4404254	*ICOS*	0,34	0,43	0,24	0,16	0,24	0,35[b]	0,35[b]	0,25	(16, 17)
rs1129055	*CD86*	0,21	0,15	0,21	0,62	0,29	0,21[c] 0,21[d]	0,20[c] 0,27[d]	0,25	(8, 36)
rs3092945	*CD40L*	0,11	0,31	0,04	0	0,01	0,21[e]	0,30[e]	0,13	(37, 38)
rs1883832	*CD40*	0,15	0,02	0,27	0,41	0,25	0,25[e]	0,16[e]	0,20	(37, 38)
rs9514828	*BLYS*	0,26	0,09	0,28	0,40	0,53	0,39[e]	0,32[e]	0,28 0,30	(37, 38)

1000Genome populations: AFR = Africans; AMR = Amerindians; ASN = Asian; EUR = Europeans. Brazilian population (BRZ). Euro- and Afro-Brazilians were classified by evaluation of phenotypical features and/or self-reported ancestry in the studies of [a](14); [b](16); [c](8); [d](36); [e](37). NA = Not available]

Table 4 Linkage disequilibrium analysis of the chromosome region 2q33 containing the genes *CD28*, *CTLA4* e *ICOS*

	rs35593994	**rs3116496**	**rs5742909**	**rs231775**	**rs4404254**
rs35593994		1	1	0,40	0,12
rs3116496	23686		0,74	0,58	0,11
rs5742909	161521	137835		0,73	0,47
rs231775	161888	138202	367		0,22
rs4404254	248744	225058	87223	86856	

Pairwise estimates of linkage disequilibrium D' (upper diagonal) and physical distance between SNPs (in bp) (lower diagonal)

Table 5 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

4. Conclusões

4. Conclusões

As seguintes conclusões puderam ser obtidas a partir da população estudada:

1- As frequências dos SNPs estudados no gene *CTLA4* não sofrem influência da ancestralidade genômica;
2- As frequências alélicas e genotípicas mostram variação dentre os SNPs investigados;
3- Os SNPs avaliados estão em equilíbrio de Hardy-Weinberg;
4- Os polimorfismos aqui estudados no gene *CTLA4* parecem não desempenhar um papel crucial na regulação da resposta imune, não influenciando na resposta de anticorpos contra a PvAMA-1, no níveis de IL-4 e no desenvolvimento da parasitemia na malária vivax;
5- A proporção de ancestralidade não varia entre os respondedores e não respondedores para anticorpos contra Ama-1.

5. REFERÊNCIAS

5. Referências

1. Tuteja, R. Malaria - an overview.The FEBS journal, v. 274, n.18, p. 4670-9, 2007.

2. Organização Mundial da Saúde. World malaria report 2011. Geneva (Switzerland): World Health Organization; (WHO) 2011.

3. Ribeiro E, Oliveira E, Melo M, Xavier B. Malária grave secundária a co-infecção por Plasmodium falciparum e Plasmodium ovale. Galicia Clin 2011; 73 (4): 25-28.

4. Oliveira-Ferreira J, Lacerda MVG, Brasil P, Ladislau JLB, Tauil PL, Daniel-Ribeiro CT. Malaria in Brazil: an overview. Malar J, v. 9, n. 1, 115, 2010.

5. Muller I, Galinski MR, Baird JK, Carlton JM, Dhanpat KK, Alonso PL, et al. Key gaps in the knowledge of Plasmodium vivax, a neglected human parasite. Lancet Infect Dis. 2009 Sep;9 (9) :555-66

6. Miller LH, Mason SJ, Clyde DF, McGinniss. The resistance factor to *Plasmodium vivax* in blacks: Duffy blood group genotype, FyFy. N Engl J Med, v. 295, n. 6, p. 302-4, 1976.

7. Ryan JR, Stoute JA, Amon J, Dunton RF, Mtalib R, Koros J; et al. Evidence for transmission of *Plasmodium vivax* among a Duffy antigen negative population in western Kenya. **Am J Trop Med Hyg**, v. 75, n. 4, p.575-81, 2006.

8. Ménard D, Barnadas C, Bouchier C, Henry-Haldin C, Gray LR, Ratsimbasoa A, et al. *Plasmodium vivax* clinical malaria is commonly observed in Duffy-negative Malagasy people. **Proc Natl Acad Sci U S A**, v. 107, n. 13, p. 5967-71, 2010.

9. Cavasini CE, DE Mattos LC, Couto AA, Bonini-Domingos CR, Valencia SH, Neiras WC, et al. *Plasmodium vivax* infection among Duffy antigen-negative individuals from

the Brazilian Amazon region: an exception? Trans R Soc Trop Med Hyg, v. 101, n. 10, p. 1042-4, 2007.

10. Medina TS, Costa SP, Oliveira MD, Ventura AM, Souza JM, Gomes TF, et al. 2011. Increased interleukin-10 and interferon-g levels in *Plasmodium vivax* malaria suggest a reciprocal regulation which is not altered by IL-10 gene promoter polymorphism. Malar. J. 10, 264.

11. Doolan DL, Dobaño C, Baird JK. 2009. Acquired Immunity to Malaria. Clin. Microbol. Rev., v.22, p.13-36.

12. Rummel T, Batchelder J, Flaherty P, LaFleur G, Nanavati P, Burns JM, et al. CD28 costimulation is required for the expression of T-cell-dependent cell-mediated immunity against blood-stage *Plasmodium chabaudi* malaria parasites. **Infect Immun**, v. 72, n. 10, p. 5768-74, 2004.

13. Pincerati MR, Dalla-Costa R, Pavoni DP, Petzl-Erler ML. Genetic polymorphisms of the T-cell coreceptors CD28 and CTLA-4 in Afro- and Euro-Brazilians. Int J Immunogenet 2010; 37:253-61.

14. Elias RM, Sardinha LR, Bastos KR, Zago CA, Silva AP, Alvarez JM, et al. 2005. Role of CD28 in polyclonal and specific T and B cell responses required for protection against blood stage malaria. J. Immunol. 174, 790–799.

15. Liu J, Zhang H, 2013. -1722T/C polymorphism (rs733618) of CTLA-4 significantly associated with lupus erythematosus (SLE): a comprehensive meta-analysis. Hum. Immunol. 74, 341–347.

16. Rudd CE, Schneider H. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signaling. **Nat Rev Immunol**, v. 3, n. 7, p. 544-56, 2003.

17. Sayegh MH, 1999. Finally, CTLA-4Ig graduates to the clinic. J. **Clin. Invest**. 103, 1223–1225.

18. Cheng TY, Lin JT, Chen LT, Shun CT, Wang HP, Lin MT,et al. 2006. Association of T-cell regulatory gene polymorphisms with susceptibility to gastric mucosa-associated lymphoid tissue lymphoma. J. Clin. Oncol. 24, 3483–3489.

19. Martins GA, Takodoro CE, Silva RB, Silva JS, Rizzo LV, 2004. CTLA-4 blockage increases resistance to infection with the intracellular protozoan *Trypanosoma cruzi*. J. Immunol. 172, 4893–4901.

20. Murphy ML, Cotterell SE, Gorak PM, Engwerda CR, Kaye PM, 1998. Blockade of CTLA-4 enhances host resistance to the intracellular pathogen *Leishmania donovani*. J. Immunol. 161, 4153–4160.

21. Jacobs T, Graefe SEB, Niknafs S, Gaworski I, Fleischer B. Murine malaria is exacerbated by CTLA-4 blockade. **J Immunol**, v. 169, n. 5, p. 2323–29, 2002.

22. Masteller EL, Chuang E, Mullen AC, Reiner SL, Thompson CB, 2000. Structural analysis of CTLA-4 function in vivo. J. Immunol. 164, 5319–5327.

23. Jha AN, Singh VK, Kumari N, Singh A, Antony J, Van Tong H, et al. 2012. IL-4 haplotype -590T, -34T and intron-3 VNTR R2 is associated with reduced malaria risk among ancestral indian tribal populations. PLoS One 7, e48136.

24. Sato M, Hiromatsu Y, Tanaka K, Ishisaka N, Nonaka K. FK506 inhibits phytohemagglutinin-, but not interferon-gamma-, induced HLA-DR antigen expression and accessory cell function on primary cultured human thyroid cells. Acta Endocrinol (Copenh). 1993 Dec;129(6):579-84. PubMed PMID: 7509103.

25. Bueno LL, Fujiwara RT, Soares IS, Braga EM, (2008) Direct effect of *Plasmodium vivax*recombinant vaccine candidates AMA-1 and MSP-1_{19} on the innate immune response. Vaccine. doi:10.1016/j.vaccine.2007.12.031.

26. Arévalo-Herrera M, Chitnis C, Herrera S, 2010. Current status of *Plasmodium vivax* vaccine. Hum. Vaccin. 6, 124–132.

27. Zakeri S, Sadeghi H, Mehrizi AA, Djadid ND, 2013. Population genetic structure and polymorphism analysis of gene encoding apical membrane antigen-1 (AMA-1) of Iranian *Plasmodium vivax* wild isolates. Acta Tropica. 126, 269–279.

28. Remarque EJ, Faber BW, Kocken CH, Thomas AW, 2008. Apical membrane antigen 1: a malaria vaccine candidate in review. Trends Parasitol. 24, 74–84.

29. Tarazona-Santos E, Raimond S, Fuseli S, 2007. Controlling the effects of population stratification by admixture in pharmacogenetics. In: Guilherme Suarez-Kurtz. (Editor): Pharmacogenomics in Admixed populations. Austin: Landes Bioscience.

30. Parra FC, Amado RC, Lambertuccci JR, Rocha J, Antunes CM, Pena SD. Color and genomic ancestry in Brazilians. Proc Natl Acad Sci U S A, 100(1):177-82, Jan,2003.

31. Pena SD, Di Pietro G, Fuchshuber-Moraes M, Genro JP, Hutz MH, Kehdy Fde S, et al. The genomic ancestry of individuals from different geographical regions of Brazil is more uniform than expected. PLoS One 2011; 6:e17063.

32. Cardena MM, Ribeiro-Dos-Santos A, Santos S, Mansur AJ, Pereira AC, Fridman C. Assessment of the relationship between self-declared ethnicity, mitochondrial haplogroups and genomic ancestry in Brazilian individuals. PLoS One 2013; 8:e62005.

33. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN, 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain

reaction and detection of a high prevalence of mixed infection. Mol. Biochem. Parasitol. 58, 283–292.

34. Vicentin EC, Françoso KS, Rocha MV, Iourtov D, Dos Santos FL, Kubrusly FS, et al. 2014. Invasion-inhibitory antibodies elicited by immunization with *Plasmodium vivax* apical membrane antigen-1 expressed in Pichia pastoris yeast. Infect. Immun. 82, 1296–1307. doi:10.1128/IAI.01169-13

35. Santos NP, Ribeiro-Rodrigues EM, Ribeiro-Dos-Santos AK, Pereira R, Gusmão L, Amorim A, Guerreiro JF, Zago MA, Matte C, Hutz MH, Santos SE. Assessing individual interethnic admixture and population substructure using a 48-insertion-deletion (INSEL) ancestry-informative marker (AIM) panel. Hum Mutat 2010; 31:184-90.

36. Warnes G, Gorjanc G, Leisch F, Man M, 2011. Genetics: Population Genetics. R package version 1.3.6. http://CRAN.R-project.org/package=genetics. Accessed 30 July 2013.

37. Sinnwell JP, Schaid DJ. 2009. haplo.stats: Statistical analysis of haplotypes with traits and covariates when linkage phase is ambiguous. R package version 1.4.4. http://CRAN.R-project.org/package=haplo.stats. Accessed 29 April 2013.

ANEXOS

Anexo 1 – Termo de aprovação do Comitê de Ética em Pesquisa (CEP)

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, 11 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

Anexo 2

ASTMH 63rd Annual Meeting

Polymorphism analysis of the CTLA-4 gene in *Plasmodium vivax* malaria patients from Brazilian Amazon region

Authors:
PAMELLA CRISTINA ALVES TRINDADE, GUSTAVO CAPATTI CASSIANO, FRANCIELE MAIRA MOREIRA BAPTISTA THOMAZ, ADRIANA ANTÔNIA DA CRUZ FURINI, MARCELA PETROLINI CAPOBIANCO, MARINETE MARINS PÓVOA, VALÉRIA DALTIBARI FRAGA, LUCIANA MORAN CONCEIÇÃO, RICARDO LUIZ DANTAS MACHADO.

Abstract
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 the two coding SNPs CTLA polymorphism in *P. vivax* patients and their correlation with parasitaemia and plasma IL-4 levels. A total of 188 *P. vivax* malaria patients were enrolled in the study. DNA was extracted from blood samples according to the standard procedure. 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 citokyne and peripheral *P. vivax* parasitaemia in Brazilian Amazon region. The CTLA-4 SNPs may be associated with malaria vivax in other endemic areas, but it appears to have no such effect in this studied population. The study also highlights the importance of conducting genetic association studies in different ethnic populations.
Finantial support: CNPq, Evandro Chagas Institut, FAPESPA and FAMERP

Anexo 3

ASTMH 63rd Annual Meeting

IL4 gene polymorphisms are not associated with *Plasmodium vivax* malaria in Brazil.

Authors:
TOMAZ, F. M. M. B. , FURINI, A. A. C., CASSIANO, G. C., CAPOBIANCO, M. P., PÓVOA, M. M., TRINDADE, P. C. A., FRAGA, V. D., AZEVEDO, L. R., OLIANI, S. M., CONCEIÇÃO, L. M., MACHADO, R. L. D.

Abstract

Introduction: 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 investigates 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. **Material and Methods:** 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 (The R Foundation for Statistical Computing, Vienna, Austria, available at http://www.r-project.org). **Results:** 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 plasma level ranged from 0,61 to 9,32 pg/mL. The parasitemia on the thick blood films ranged from 5 to 15.000 parasites/mm^3. There were no statistically significant differences either in parasitaemia, plasma IL4 level among individuals with different genotypes and haplotypes. **Conclusion:** Our findings suggest that IL4 gene polymorphisms were not associated with serum citokyne and peripheral *P. vivax* parasitaemia in Brazilian Amazon region. The present findings reinforce and increase our understanding about the role of the immune system in on the clinical course of the severe malaria vivax.

Finantial support: CNPq and Evandro Chagas Institut and FAMERP.

Anexo 4

ASTMH 63rd Annual Meeting

Study on association between genetic polymorphims of tumour necrosis factor-α, interleukin-10, interferon-γ, and malaria vivax in Brazil.

Authors:
Adriana C. Furini[1], Gustavo C. Cassiano[1,2], Franciele M. B. Tomaz[1], Marcela P. Capobianco[1,2], Marinete Marins Póvoa[3], Michele Encinas [4], Diego Longo Madi[4], Pamella C. A. Trindade[1], Valeria Daltibari Fraga[1], Luciana Moran Conceição[1], Ricardo L. D. Machado[1,2,3]

[1]Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, Brasil, [2]Universidade Estadual Paulista, São José do Rio Preto, Brasil, [3] Instituto Evandro Chagas, Ananindeua, Brasil, [4]Centro Universitário de Rio Preto, São José do Rio Preto, Brasil. E-mail: adriana.cruz.furini@gmail.com

Introduction: 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*, *IFNγ*, *TNFα* 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. **Material and Methods:** 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. **Results:** All polymorphisms tested were in Hardy-Weinberg equilibrium. The African, European and 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. **Conclusion:** These findings make us to believe that the analyzed polymorphisms are not associated with susceptibility or resistance with vivax malaria in the studied population.

Financial support: CNPq and Evandro Chagas Institut and FAMERP

Anexo 5

49ª edição do Congresso da Sociedade Brasileira de Medicina Tropical.
ÁREA TEMÁTICA: F) DOENÇAS POR PROTOZOÁRIOS - PAINEL: 752

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

Autores:
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

Resumo

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 *IFNgama*, *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 mm3. 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.

Anexo 6

49ª edição do Congresso da Sociedade Brasileira de Medicina Tropical.

ÁREA TEMÁTICA: F) DOENÇAS POR PROTOZOÁRIOS

POLIMORFISMOS NO GENE DA *IL4* NÃO INFLUENCIAM A PARASITEMIA NA MALÁRIA VIVAX NÃO GRAVE NO BRASIL.

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

Instituição:
FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO

Resumo

Na malária vivax o balanço entre as citocinas pró e antiinflamatórias é necessário para o controle das alterações imunopatológicas. A proteína expressa pelo gene *IL4* ativa as células B, com diminuição da resposta tipo Th1, além de estimular o crescimento e diferenciação de eosinófilos. O SNP na região promotora dessa citocina, pode afetar os níveis de transcrição do mesmo, e a variante alélica T está associada a maiores níveis de IgE em pacientes com malária grave. Nós investigamos as frequências alélicas e genotípicas nas posições (-590 C>T) e um VNTR no íntron 3 desse gene, em 85 indivíduos com malária vivax residentes no município de Goianésia do Pará. A extração de DNA foi utilizada empregando-se o kit de extração/purificação Easy-DNA™ (Invitrogen, Califórnia – USA) 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. Os dados revelam uma frequência maior para o genótipo CT (54,3%) para o gene da *IL4* (– 590C>T) seguido do genótipo CC (28,6%) e uma frequência menor para o genótipo TT (17,1%). Para o VNTR o genótipo mais frequente foi B1/B2 (50%) seguido do genótipo B2/B2 (41,7%), enquanto que para o genótipo B1/B1 foi observado uma baixa frequência (8,3%). Nossos resultados mostraram que para o SNP -590 C>T e a repetição em *TANDEM* VNTR não houve diferença estatística significante. Entretanto os indivíduos com

genótipo TT (-590) apresentaram maior parasitemia (p=0,06). Os polimorfismos no gene *IL4*, que codificam molécula que interagem em vias moduladoras da resposta imune humoral, podem contribuir para níveis alterados da citocina, nos níveis de anticorpos e consequentemente na carga parasitária e, portanto, influenciar na susceptibilidade a malária. Dessa forma, os resultados obtidos poderão contribuir na identificação e participação efetiva de genes humanos na modulação da resposta imune, essenciais no estabelecimento de estratégias de imunização contra a doença, em área de transmissão ativa localizada no Estado do Pará, com o objetivo de produzir informações e possíveis soluções para o controle desta endemia.

Fonte Financiadora: FAMERP e CNPq

Anexo 7

49ª edição do Congresso da Sociedade Brasileira de Medicina Tropical.

ÁREA TEMÁTICA: F) DOENÇAS POR PROTOZOÁRIOS

POLIMORFISMOS EM GENES CO-ESTIMULATÓRIOS NÃO INFLUENCIAM A RESPOSTA DE ANTICORPOS CONTRA A MSP-1_{19} DO *Plasmodium vivax* EM INDIVÍDUOS NATURALMENTE INFECTADOS EM UMA ÁREA ENDÊMICA DA AMAZÔNIA BRASILEIRA.

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, RICARDO LUIZ DANTAS MACHADO

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 7 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%. Apesar de não ter sido detectada associação estatisticamente significativa, os indivíduos com o genótipo TT para o SNP rs1883832 no gene *CD40* apresentaram maiores índices de reatividade de anticorpos ($p = 0,06$). Os indivíduos apresentando no mínimo um alelo A no SNP rs1129055 no gene *CD86* também apresentaram maiores índices de reatividade de anticorpos contra a MSP-1_{19} ($p = 0,08$). Não houve associação significativa entre os genótipos estudados e a frequência de respondedores. **Discussão:** 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*. No entanto, devido a óbvia importância dos sinais co-estimulatórios na malária, maiores estudos deverão ser realizados para avaliar se estes polimorfismos influenciam a produção de anticorpos contra outros antígenos do parasito. **Conclusão:** Os nove SNPs investigados (rs9514828, rs35593994, rs3116496, rs1883832, rs3092945, rs1129055, rs5742909, rs231775 e rs4675378) não influenciaram a resposta de anticorpos contra a MSP-1_{19} do *P. vivax* em uma população da Amazônia brasileira.

Apoio financeiro: Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq).