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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Sample

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

2.2. Genotyping

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

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

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

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

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

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

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

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

2.3. Determination of ancestry

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

2.4. Statistical analysis

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

3. Results

3.1. Distribution of polymorphisms according to ancestry

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

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

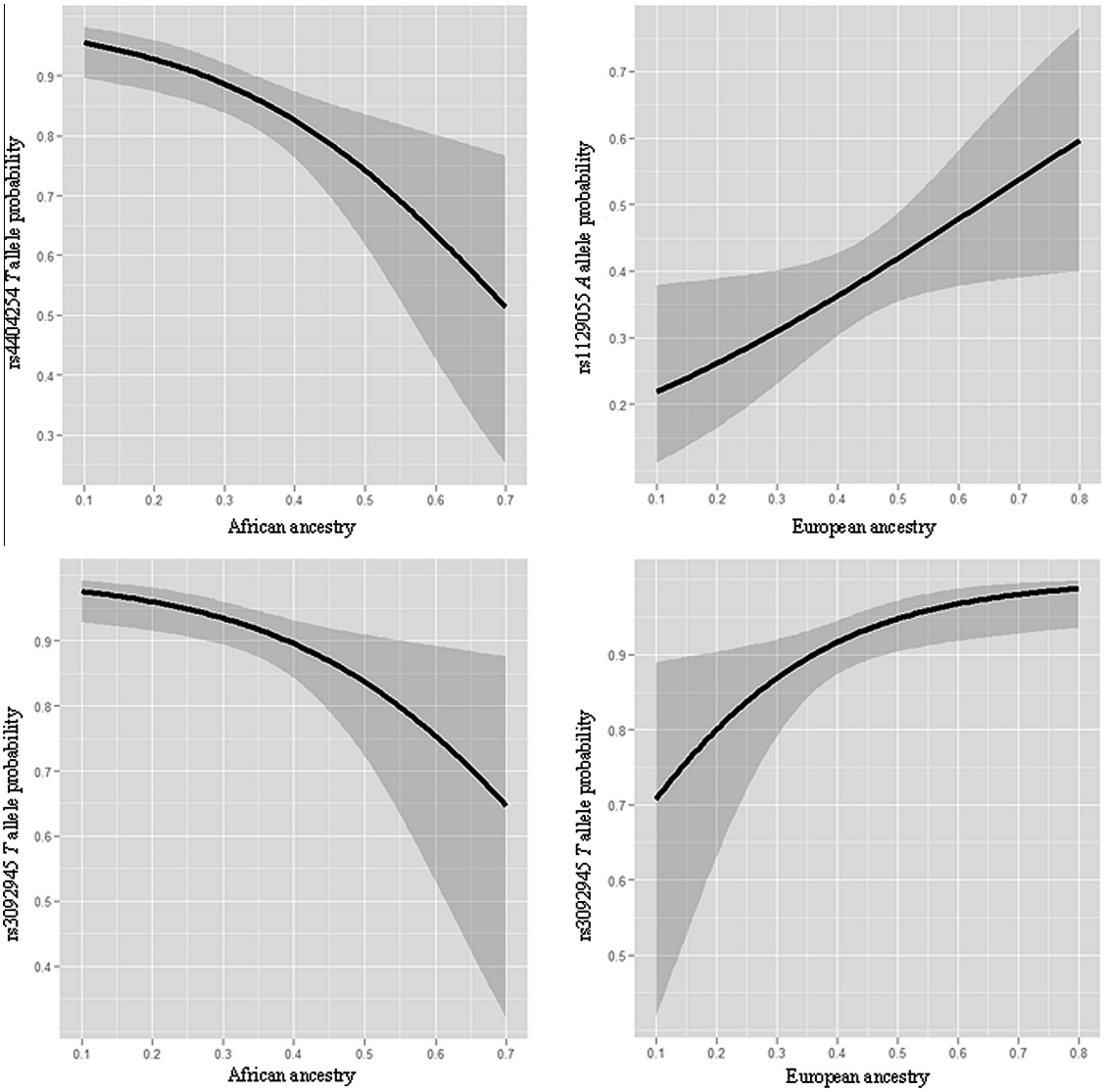

Fig. 1. Fitted logistic regression describing the association between ancestry and polymorphisms. (a) *ICOS* rs4404254. Chance of having a *T* allele according to African ancestry. (b) *CD86* rs1129055. Chance of having a *A* allele according to European ancestry. (c) *CD40L* rs3092945. Chance of having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

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

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

3.2. Linkage disequilibrium and haplotypes

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

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

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

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

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

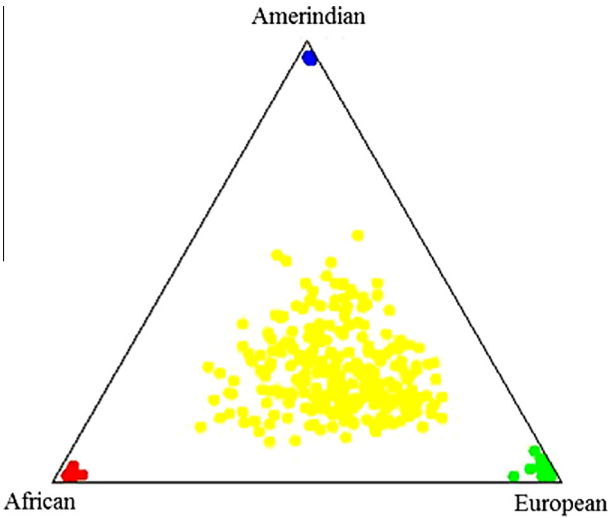

Fig. 2. Schematic representation of the individual admixture estimates. Each point represents one individual and the correspondent admixture proportions are indicated by the distance to the edges of the triangle. European, African and Amerindian correspond to individuals from the parental populations.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

3.3. Estimate of ancestry

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

4. Discussion

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

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

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

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

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

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

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

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

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