**1. Introduction**

The three non-classical class I b genes, HLA-E, HLA-F and HLA-G, located in the class I region of the Major Histocompatibility Complex (MHC) in humans can be distinguished from the classical class I genes by their low allelic polymorphism, specific expression patterns, peptide binding properties and immune-related functions [1]. The recent description of HLA-E and HLA-G antigens as ligands for distinct killing inhibitory receptors expressed by immunocompetent cells has highlighted the specific role of non-classical class I molecules in modulating immune responses. In contrast to HLA-C, which has a restricted pattern of expression [2], HLA-E is ubiquitously expressed [3]. HLA-E is located between the HLA-C and -A genes [4] and, unlike MHC class Ia, is highly conserved and the biological significance of its genetic polymorphism is uncertain [5]. Currently, 11 alleles are described in the literature: HLA-E (E\(^\text{01:01:01:01}, E\(^\text{01:01:01:02}, E\(^\text{01:01:03:01}, E\(^\text{01:03:01:02}, E\(^\text{01:03:02:01}, E\(^\text{01:03:03:02}, E\(^\text{01:03:03:03}, E\(^\text{01:03:03:04}, E\(^\text{01:03:05}, E\(^\text{01:04}), which encode only three protein products (IMGT/HLA Database) [6]. There are three allelic variations which are non-synonymous and correspond to E\(^\text{01:01}, E\(^\text{01:03} and E\(^\text{01:04}) [7]. Alleles HLA-E\(^\text{01:01}, HLA-E\(^\text{01:03} and HLA-E\(^\text{01:04} differ only at codon 107 of exon 3; HLA-EG presents codon (GGG) which stands for asparagine whereas HLA-ER has an arginine (AGG) [8]. These two alleles seem to have different levels of expression and HLA-EG molecules are found in a higher concentration in the cell surface as compared to HLA-ER [9]. This difference in expression of the two alleles not only reflects the affinity of HLA-E107G for peptides but also the greater stability of the complex on the cell surface. The variation E\(^\text{01:04} has two nucleotide substitutions: the first is located at codon 107 which encodes a glycine (GGG) instead of an arginine (AGG). The second at codon 157 which presents a substitution of an arginine for a glycine, differentiates these alleles from the afore mentioned.

The HLA-E molecule shows less variation in its amino acid sequence than other HLA class I molecules [10]. For the stabilization of the molecule on the cell surface, peptides derived from the leader sequence of several HLA class I molecules including HLA-A, HLA-B, HLA-C and HLA-G are requested [11]. Evidences suggest that HLA-E molecules can also bind to viral and bacterial peptides [12]. HLA-E has been identified as a ligand for the CD94/NKG2A and CD94/NKG2C receptors expressed in NK cells, which are part of innate immunity [13]. This interaction is dependent on the association of HLA-E with nonamers derived from class I leader sequence and variations in these peptides affect the stability of HLA-E determining whether HLA-E/peptide complexes are stable and can be expressed in the cell surface and can protect this cell from NK cell-mediated killing.
from NK lysis after being recognized by the CD94/NKG2A receptors [14]. Interestingly, the presence of HLA-E at the cell surface serves as a surrogate marker for the integrity of the key parts of the class I antigen processing pathway. Any interference of processing or of display will render the cell vulnerable to lysis by NK cells [15].

In this study we analyzed polymorphisms in exons 2 and 3 of the HLA-E gene in a sample of Afro-descendants who live in the state of Paraná, in Southern Brazil. The aim of this work was to characterize these gene polymorphisms in our population and to present data which can serve as a basis for future population and association studies. Few population studies have been conducted for HLA-E and in only a limited number of populations. The importance of HLA-E variation to transplant rejection, disease susceptibility and immunity has yet to be explored.

2. Materials and methods

2.1. Study population

A total of 152 unrelated healthy bone marrow donors were genotyped for HLA-E exons 2 and 3 at the Laboratory of Immunogenetics and Histocompatibility of the Federal University of Paraná, Brazil (LIGH–UFPR). All individuals classified themselves as Afro-Brazilians and were mainly from the capital city of the State of Paraná, Curitiba and from its metropolitan region. Samples were collected from 2007 to 2010. The study was approved by the Ethics Committee of the Hospital das Clínicas of the UFPR (HC–UFPR/CPE/C no. 037 ext.019/2001–07). All participants signed a free and informed consent form and completed a personal and occupational questionnaire.

2.2. HLA-E genotyping

Exons 2 and 3 of the HLA-E gene were amplified by PCR using oligonucleotide primers (5′ GGGTCGGATGGGAAACCGGC 3′) and (5′ GTTCCGACCTTGTTGCTGTA 3′) for exon 2 and (5′ CCGAA CCCGCCAGACCCCTA 3′) and (5′ TGAGGTCGTACGTGTGGG 3′) for exon 3. PCR was performed according to the following conditions: final volume of 50 μL containing: 1X PCR buffer (70 mM Tris–HCl pH8.8, 20 mM(NH4)2SO4), 1 mM MgCl2, 0.45 mM of each dNTPs, 20 pMol of each primer, 2 units of Taq DNA polymerase Platinum (Invitrogen, Carlsbad, CA) and 30 ng of genomic DNA, resulting in a 985 bp PCR product. The cycle of initial denaturation was 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 60 s. The final extension step was 72 °C for 4 min (GeneAmp PCR System 9700) 12 μL of PCR product were then purified, using 20 units of Exonuclease (EXOI) (United States Biochemical – USB, Staufen, Germany), 4 units of shrimp alkaline phosphatase (SAP) (USB) and 2× SAP Buffer (USB). The purified PCR product of exons 2 and 3 of the HLA-E gene were sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Kit version 3.3 (Applied Biosystems, CA, USA) and ABI PRIMERS 3130 Genetic Analyzer (Applied Biosystems, CA, USA). The primers used in the direct DNA sequencing reaction were the same as for PCR amplification (forward and reverse). All the sequences obtained from each sample were aligned with the genomic sequences of the official alleles (recognized by the WHO and the International Immunogenetics Information System – IMGT) and each single nucleotide polymorphism (SNP) detected was individually noted.

2.3. Statistical analysis

Allele and genotype frequencies were determined by direct counting. Adherence of genotype proportions to expectations under Hardy–Weinberg equilibrium were tested by the exact test of Guo and Thompson using the GENEPOP 3.4 software. Haplotype frequencies and LD values were calculated with the software package Arlequin version 3.1 [16]. Genetic similarity between our sample and different ethnic groups were calculated with program Biostat version 5.0 and the analysis of molecular variance (AMOVA) used Arlequin for values of FST. Departure from selective neutrality was evaluated by three different methods: the Ewens–Watterson test implemented by Slatkin that permits to test alternative hypotheses of either directional or balancing selection; the Tajima’s D test, which examines the relationship between the number of segregating sites and nucleotide diversity by comparing the sequence diversity statistics αn and π and the Fu and Li test which included the synonymous and non-synonymous nucleotide substitutions. All tests were carried out using the ARLEQUIN version 3.1 program.

3. Results

Allele, genotype and haplotype frequencies found in the sample population studied are shown in Table 1. E01:01:03:02 and E01:03:04 alleles were considered together as exon 8 was not analyzed. The E01:04 allele was not found in our sample. Thus, it was not possible to confirm its existence, corroborating the hypothesis that this allele may be an artifact of sequencing. On the other hand the allele with the highest frequency was E01:01 (59.1%). All genotype frequencies were in Hardy–Weinberg equilibrium, with P = 0.3389. We report only the haplotypes that were found in a frequency superior to 0.04%. They were used for the analysis of molecular variance in order to compare genetic diversity between the different populations studied. According to the results obtained by AMOVA, the degree of differentiation between our sample and Euro-Caucasians studied by Antoun et al. [17], was 3.874%, P = 0.035. In turn, the difference obtained between our population and Afro-Americans reported by Grimsley and Ober [18] was of 5.368% with P = 0.069. These data show that our sample is more similar to the Euro-Caucasians due to the European migration to our country. Neutrality tests did not present significant results when exons were examined together or separately.

4. Discussion

The analysis of genetic polymorphisms in exons 2 and 3 of the HLA-E gene in a sample composed by self-reported Afro-descendants living in the southern state of Paraná, Brazil allows us to infer that the frequencies of the E01:01 and E01:03 alleles are similar, reinforcing the possibility of balanced selection. The HLA-E01:04 allele was not found in our sample.

The genotype frequencies of SNPs 1114 and 1446 were compared between our sample and results found by Antoun et al. in Euro-Caucasian, African Caribbean and Asian populations (data not shown) [17]. Differences in the genotype SNPs frequencies were not significant when our sample was compared with Euro-Caucasians and African Caribbeans. In turn, statistically significant differences were found between our population and Asians analyzed by the same authors. These results are expected due to the ethnic composition of our sample which was formed by Africans who came to our country as working force and admixed with Euro-Caucasians and the native indigenous populations.

We compared the similarity of frequencies of alleles E01:01; E01:03:01, E01:03:03 and E01:02:02:01 between our sample and healthy bone marrow donors from the state of São Paulo, Brazil, Caucasians of Denmark and African Americans of the United States (Table 2) [18,19]. Interestingly our sample differed signifi-
cantly from the sample of individuals from São Paulo, studied by Veiga-Castelli et al. [20]. This reflects a different population composition between both samples. Our sample also differed significantly from the Caucasian sample of Denmark and was similar to the African American sample studied by Stefensen et al. [19].

Our allele frequencies were also compared with three different Amerindian samples from Mexico, Colombia and Chile [21]. Significant differences were observed in all instances which can be explained by the fact that we are comparing an Afro-descendant sample with the indigenous Americans. Even if our sample was expected to have an Ameridian component due to admixture, it was not relevant due to the fact that native settlements were underrepresented in our region.

Comparisons between the following genotype frequencies (\( E'01:01/01:01; E'01:01/01:03; E'01:03/01:03 \)) obtained in our sample with data reported in the literature [18] were significantly different only when the Chinese population was considered (Table 2). This result is widely expected due to the fact that the Chinese did not participate in the ethnic formation of the Brazilian nation.

Our frequencies were statistically similar to those found in African Americans and Caribbeans. Significant differences were observed between our sample and results reported in the literature for Chinese and other Ameridian populations. Through the calculation of the Fst it was possible to conclude that the Afro-descendants from Paraná are genetically more similar to Euro-Caucasians when compared to Afro-Americans. These data may reflect the different African origins between our Afro-descendants, who arrived to Brazil as slaves, brought by our Portuguese colonizers and Afro-Americans. The latter can have their origins traced to what is today Senegal and Gambia whereas Afro-Brazilians came mostly from Nigeria, Benin, Angola and Mozambique [22]. It is very well established and known that the African continent is multiethnic with very different populations from the genetic as well as from the cultural point of view. Our Southern Brazilian population has an important European contribution, of approximately 80%, and this component is nowadays seen also in our Afro-descendants[23].

In conclusion, this is the first study regarding HLA-\(E\) polymorphisms in a Southern Brazilian urban population composed of self-reported Afro-descendants performed at such refined resolution level. Our results reflect our ethnic history, composition and evolution. Results obtained with the different selective neutrality tests were not significant and further studies would be interesting to confirm results obtained by Veiga-Castelli et al. [20] regarding the different selective pressures that may be acting in the different exons and intronic regions.

### Table 1

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Allele frequencies in Afro-descendants from Paraná.</th>
<th>n = 304</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>E'01:01</td>
<td>180</td>
<td>59.1</td>
<td></td>
</tr>
<tr>
<td>E'01:03:01</td>
<td>38</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>E'01:03:02/01:03:04</td>
<td>76</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>E'01:03:03</td>
<td>10</td>
<td>3.4</td>
<td></td>
</tr>
</tbody>
</table>

**Genotypes**

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Genotype frequencies in Afro-descendants from Paraná.</th>
<th>n = 152</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>E'01:01, 01:01</td>
<td>56</td>
<td>36.84</td>
<td></td>
</tr>
<tr>
<td>E'01:01, 01:03:01</td>
<td>26</td>
<td>17.10</td>
<td></td>
</tr>
<tr>
<td>E'01:01, 01:03:02/01:03:04</td>
<td>42</td>
<td>27.63</td>
<td></td>
</tr>
<tr>
<td>E'01:03:01, 01:03:01</td>
<td>5</td>
<td>3.29</td>
<td></td>
</tr>
<tr>
<td>E'01:03:01, 01:03:02/01:03:04</td>
<td>12</td>
<td>7.89</td>
<td></td>
</tr>
<tr>
<td>E'01:03:02/01:03:04, 01:03:04</td>
<td>8</td>
<td>5.26</td>
<td></td>
</tr>
<tr>
<td>E'01:03:02/01:03:04, 01:03:03</td>
<td>3</td>
<td>1.97</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2

**Allele and genotype frequencies comparisons between four different ethnic groups.**

<table>
<thead>
<tr>
<th>Alleles</th>
<th>ADP</th>
<th>BRA</th>
<th>CAD</th>
<th>AEUA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele</td>
<td>n</td>
<td>Freq.%</td>
<td>n</td>
<td>Freq.%</td>
</tr>
<tr>
<td>E'01:01</td>
<td>180</td>
<td>59.21</td>
<td>65</td>
<td>62.50</td>
</tr>
<tr>
<td>E'01:03:01</td>
<td>38</td>
<td>12.5</td>
<td>10</td>
<td>9.13</td>
</tr>
<tr>
<td>E'01:03:03</td>
<td>10</td>
<td>3.28</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>E'01:03:02/01:03:04</td>
<td>76</td>
<td>25.00</td>
<td>25</td>
<td>24.52</td>
</tr>
<tr>
<td>E'01:01, 01:03</td>
<td>38</td>
<td>12.5</td>
<td>10</td>
<td>9.13</td>
</tr>
<tr>
<td>E'01:03:01, 01:03:02/01:03:04</td>
<td>12</td>
<td>7.89</td>
<td>10</td>
<td>9.13</td>
</tr>
</tbody>
</table>

**Genotypes**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>ADP</th>
<th>AEUA</th>
<th>HI</th>
<th>CH</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Freq.%</td>
<td>n</td>
<td>Freq.%</td>
<td>n</td>
</tr>
<tr>
<td>E'01:01, 01:01</td>
<td>56</td>
<td>37</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>E'01:01, 01:03</td>
<td>68</td>
<td>45</td>
<td>10</td>
<td>45</td>
</tr>
<tr>
<td>E'01:03:01, 01:03:03</td>
<td>28</td>
<td>18</td>
<td>13</td>
<td>19</td>
</tr>
</tbody>
</table>

Source: Modified Stefensen et al. [19]. Data obtained from the author: CAD, AEUA, Veiga-Castelli et al. [20]. Data obtained from the author: BRA, Grimsley and Ober [18]. Data obtained from the author: AEUA, HI and CH. Allele frequencies: Statistical difference between ADP and BRA, \( \chi^2 = 55.5868, P < 0.003; \) Statistical difference between ADP and CAD, \( \chi^2 = 67.5316, P < 0.001; \) Statistical difference between ADP and AEUA, \( \chi^2 = 5.014, P = 0.0815. \) Genotype frequencies: Statistical difference between ADP and AEUA, \( \chi^2 = 0.037, P = 0.9816, \) Statistical difference between ADP and HI, \( \chi^2 = 1.367, P = 0.50491. \) Statistical difference between ADP and CH, \( \chi^2 = 20.077, P < 0.0001. \)
Acknowledgements

This study was supported by the LIGH-FUNPAR Alliance Research Fund. We are very grateful to the volunteer bone marrow donors for generously accorded to provide samples for this study. We also thank LIGH staff for technical support.

References