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# HIGH-RESOLUTION HLA-DRB1 ALLELE FREQUENCIES IN A ROMANIAN COHORT OF STEM CELL DONORS

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## ABSTRACT

The goal of the current study was to determine the high-resolution frequencies of the HLA-DRB1 alleles among the analyzed Romanian cohort of healthy stem cell donors. Using Next Generation Sequencing (NGS), we estimated class II HLA-DRB1 allele frequencies to a 6-digit resolution through HLA typing in a Romanian cohort of healthy individuals. The study for HLA genotyping included 420 willing donors from the National Registry of Voluntary Hematopoietic Stem Cell Donors (RNDVCSH). In 2020 and 2021, peripheral blood samples were collected and transported to the Fundeni Clinical Institute. We used the Immucor Mia Fora NGS MFlex kit for HLA genotyping. Forty-one different alleles were detected in 420 analyzed samples, out of which the most frequent HLA-DRB1 alleles were DRB1*16:01:01 (12.6%), DRB1*11:04:01 (12.1%) and DRB1*03:01:01 (12%). The HLA-DRB1*11:01:02 and -DRB1*08:04:01, -DRB1*05:01:01, -DRB1*13:05:01, -DRB1*14:07:01, -DRB1*09:01:02, -DRB1*11:02:01, -DRB1*04:07:01, -DRB1*15:03:01, -DRB1*03:02:01, -DRB1*04:06:02, -DRB1*04:08:01, -DRB1*14:05:01 were identified only once. The results revealed similarities with countries belonging to the Eastern Europe, the Balkans and the Caucasus regions. Further studies on larger Romanian cohorts are needed for confirming the current results.

**Keywords**: HLA-DRB1, allele frequencies, transplantation immunogenetics

## INTRODUCTION

The Major Histocompatibility Complex (MHC) has been the subject of much scientific interest in recent years especially due to the numerous studies on the HLA genes, part of the MHC [1-2]. The human leukocyte antigen system (HLA) is one of the most polymorphic genetic systems in the human genome [1,3]. Accurate HLA allele identification is essential for both anthropological research and for the field of organ and stem cell transplantation. Because more and more HLA alleles are being discovered through multiple studies, Next Generation Sequencing (NGS) HLA genotyping is necessary [3-4]. Using NGS for HLA typing has two advantages, one being able to resolve allelic ambiguities and the other in establishing updated allele frequencies [3-4]. These advantages will support the use of HLA types in research and clinical medicine in more precise and thorough ways [1-4].

HLA-DRB1 is one of HLA class II's beta chain paralogues. Alpha (DRA) and beta (DRB), both of which are anchored in the membrane, form the heterodimer that is the class II molecule [5-7]. Presenting peptides derived from extracellular proteins, it plays a pivotal role within the immune system. Antigen-presenting cells, or APCs (B lymphocytes, dendritic cells, and macrophages), express class II molecules [6-10]. Professional antigen-presenting cells (APCs) in complex with the alpha chain HLA-DRA present antigenic peptides for recognition by the alpha-beta

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T cell receptor (TCR) on HLA-DRB1-restricted CD4-positive T cells [6-10]. This directs the actions of T-helper effectors that are specific to antigens, thereby facilitating the elimination of infectious agents and transformed cells through antibody-mediated immune response and macrophage activation [8-10].

Genetic variants have been proven to play an essential role in most of the common human disorders (i.e. obesity, type II diabetes, hypertension, neurological disease, cancer, etc.) [11-12], research conducted on HLA-DRB1 frequencies on a variety of populations has also linked HLA-DRB1 alleles with the susceptibility and clinical response for many disorders, such as rheumatoid arthritis, sarcoidosis, Goodpasture syndrome and multiple sclerosis [13–20]. In order to comprehend the risk for these disorders, it is crucial first to determine the frequencies of the various HLA-DRB1 alleles in the Romanian population.

The goal of the current study was to determine the high-resolution frequencies of the HLA-DRB1 alleles among a healthy Romanian cohort of healthy stem cell donors whose data was analyzed.

## MATERIAL AND METHODS

The study for HLA typing included 405 healthy voluntary donors (Romanians/Europeans, 61% male, age 43.3 ± 7.7 years) who were registered in the National Registry of Voluntary Hematopoietic Stem Cell Donors (RNDVCSH).

This study was conducted at the Fundeni Clinical Institute in the Medical Analysis Laboratory 2, and it involved healthy donors who voluntarily registered for stem cell donation in the RNDVCSH between 2020 and 2021.

In accordance with the Declaration of Helsinki, written consent from willing donors was requested for the processing of personal data and evidence. This study was reviewed and approved by the Ethics Committee of the Fundeni Clinical Institute (no. Ten Points: 7916/10.02. 2021).

Each donor's medical file contained medical data that the project's research team extracted, processed, and statistically examined. We included willing donors between the ages of 20 and 50 in the study who had no underlying health issues. We looked through each donor's personal medical record to see if their medical history was in compliance with the national protocol. After they donated blood, we also looked at their viral status and biochemical parameters.

Peripheral blood collected in vacutainers containing the anticoagulant EDTA (ethylene-diamino-tetra-acetic acid) provided the DNA used in this investigation. DNA was extracted from blood using the manual technique. DNA was extracted using the QIAmp DNA Blood Mini® extraction kit (QIAGEN, Hilden, Germany). The purification of total DNA (genomic, mitochondrial) from bone marrow, cell cultures, leukocyte concentrate, and whole blood was made possible by this rapid and easy technique based on silicon dioxide membranes.

Each blood sample was thoroughly vortexed, mixed with protease and lysis buffer, and heated in a thermoblock for 10 minutes at 56 degrees Celsius to facilitate rapid lysis. The DNA was still free in the lysate after the cell membranes broke down, so we added 80% alcohol to make it precipitate. The lysate was placed into tubes with silicon membranes, to which DNA adheres, because the two materials had different electrical charges. The DNA was purified by multiple washings and separated from the silicon membrane following the addition of the elution buffer, which neutralizes the electrical charges.

Prior to usage, the DNA was divided into tubes and stored at -18 °C. An IMPLEN nanophotometer was used to measure the concentration and purity of the DNA using an A260nm/A280nm ratio between 1.7 and 1.9, certifying solution purity and a DNA concentration at >20 ng/µL.

The Mia Fora NGS MFlex kit from Immucor was used to genotype the HLA class II alleles (HLA-DRB1, DRB3/4/5) at a 6-digit resolution. Using Next-Generation Sequencing techniques, HLA genotyping was carried out using the MIA FORA NGS MFlex HLA kit (MIA FORATM NGS MFlex) from Immucor. The three main components of this process are long-range PCR, library construction, and sequencing and data analysis. In the long-range PCR step, the most pertinent HLA genes were amplified. After fragmented probes are used to build libraries, adenine nucleotides are added to the ends of each fragment to enhance the ligation of the unique index adapters. Each fragment is barcoded to make identification easier during sequencing. To ensure adequate cluster generation, a final amplification of the size-selected library was then required. By using the Pippin Prep system, DNA fragments containing 500–900 base pairs were selected. Before the final library preparation, the concentration was measured using a Qubit® fluorometer (Thermo Fisher Scientific) and adjusted according to the protocol.

Using Illumina reagents, the NGS sequencing library was prepared and then loaded into an Illumina MiniSeq sequencer. Making use of the MIA FORA NGS FLEX program (Sirona Genomics, Inc. Sirona Genomics and IMGT databases, two reference databases, were used to interpret data after sequencing was completed. To confirm the allele frequency distribution among the analyzed population, the Hardy-Weinberg equilibrium was used.

## RESULTS

A total of 41 different HLA-DRB1 alleles were detected in our cohort (table 1).

**Table 1.** The alleles with the highest frequencies for the HLA-DRB1 locus were represented by the DRB1*16:01:01 (12.6%), DRB1*11:04:01 (12.1%), DRB1*03:01:01 (12%), DRB1*07:01:01 (9.3%), DRB1*01:01:01 (7.3%), DRB1*11:01:01 (6.3%) and DRB1*13:01:01 (6%) (table 1). Out of the total of 41 detected alleles, 3 had frequencies higher than 10% (table 1), identified out of a total of 297 individuals. The top 3 alleles amount to more than one third of all the identified alleles (36.67%) (table 1). All these 3 alleles had frequencies of 12% - 13% [DRB1*16:01:01 (12.6%), DRB1*11:04:01 (12.1%), DRB1*03:01:01 (12%)], with the top frequency at 12.6% (table 1, figure 1).7 of the 41 HLA-DRB1 alleles had frequencies higher than 5%.

**Table 1.** HLA-DRB1 alleles identified in the Romanian cohort (6-digits)

| HLA-DRB1 alleles | No. | AF (%) |
|---|---|---|
| *16:01:01 | 102 | 12.59 |
| *11:04:01 | 98 | 12.10 |
| *03:01:01 | 97 | 11.98 |
| *07:01:01 | 75 | 9.26 |
| *01:01:01 | 59 | 7.28 |
| *11:01:01 | 51 | 6.30 |
| *13:01:01 | 49 | 6.05 |
| *15:01:01 | 35 | 4.32 |
| *13:02:01 | 24 | 2.96 |
| *04:01:01 | 21 | 2.59 |
| *04:02:01 | 17 | 2.10 |
| *04:03:01 | 17 | 2.10 |
| *13:03:01 | 15 | 1.85 |
| *14:54:01 | 15 | 1.85 |
| *10:01:01 | 14 | 1.73 |
| *15:02:01 | 13 | 1.60 |
| *04:04:01 | 11 | 1.36 |
| *16:02:01 | 11 | 1.36 |
| *12:01:01 | 10 | 1.23 |
| *08:01:01 | 9 | 1.11 |
| *01:02:01 | 9 | 1.11 |
| *14:03:01 | 9 | 1.11 |
| *14:01:01 | 9 | 1.11 |
| *04:05:01 | 8 | 0.99 |
| *14:04:01 | 8 | 0.99 |
| *11:03:01 | 6 | 0.74 |
| *08:03:02 | 3 | 0.37 |
| *02:02:01 | 2 | 0.25 |
| *11:01:02 | 1 | 0.12 |
| *08:04:01 | 1 | 0.12 |
| *05:01:01 | 1 | 0.12 |
| *13:05:01 | 1 | 0.12 |
| *14:07:01 | 1 | 0.12 |
| *09:01:02 | 1 | 0.12 |
| *11:02:01 | 1 | 0.12 |
| *04:07:01 | 1 | 0.12 |
| *15:03:01 | 1 | 0.12 |
| *03:02:01 | 1 | 0.12 |
| *04:06:02 | 1 | 0.12 |
| *04:08:01 | 1 | 0.12 |
| *14:05:01 | 1 | 0.12 |
| **Total general** | **810** | **100** |

AF – Allele Frequency; No. – Number of alleles

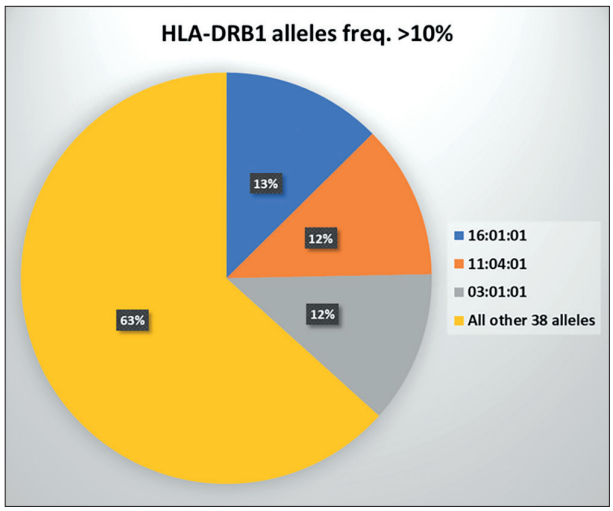


**Figure 1.** HLA-DRB1 alleles with frequencies > 10% (6-digits)

In total, the added frequencies of these 7 alleles represent almost two thirds (65.55%) of all detected HLA-DRB1 variants, identified in 531 individuals. HLA-DRB1 alleles with low frequencies (<5%) stand for 35% (279 individuals) of all observed variants (table 1).

Out of the entire cohort and all detected HLA-DRB1 variants, 18 alleles showed frequencies lower than 1% (table 1). 13 of these rare variants were detected in just one individual each (0.12%), while the HLA-DRB1*02:02:01 allele was observed in 2 individuals (0.24%) and DRB1*08:03:02 in 3 (0.37%), all with frequencies lower than 0.5%.

3 of the 18 rare alleles had frequencies of 0.5%-1% (DRB1*11:03:01 and DRB1*14:04:01, both in 8 individuals, 0.99% each) and DRB1*11:03:01 (6 persons, 0.74%).

Apart from the aforementioned 18 rare HLA-DRB1 alleles, 4 others had frequencies close to 1% (1.11%), being detected in 9 individuals each. These 4 alleles represent together 4.44% of all resulted HLA-DRB1 variants.

In total, 22 HLA-DRB1 alleles had frequencies of a maximum of 1.11%, totalizing 9.38% of all detected variants and being identified in 76 individuals. The rest of the 734 HLA-DRB1 alleles represent 90.62% of all variants.

When adding the identified frequencies of all the variants for each HLA-DRB1 allele, the top alleles, with

**Table 2.** HLA-DRB1 allele frequencies (2-digits)

| HLA-DRB1 alleles | AF (%) |
|---|---|
| **DRB1*11** | 19.38 |
| **DRB1*16** | 13.95 |
| **DRB1*03** | 12.10 |
| **DRB1*13** | 10.99 |
| **DRB1*04** | 9.51 |
| **DRB1*07** | 9.26 |
| **DRB1*01** | 8.40 |
| **DRB1*15** | 5.93 |
| **DRB1*14** | 5.31 |
| **DRB1*10** | 1.73 |
| **DRB1*12** | 1.23 |
| **DRB1*02** | 0.25 |
| **DRB1*05** | 0.12 |
| **DRB1*09** | 0.12 |

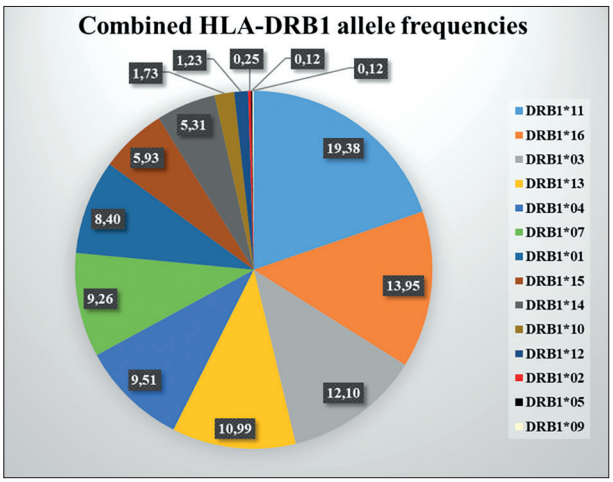


**Figure 2.** Combined HLA-DRB1 allele frequencies (2-digits)

frequencies higher than 10%, were DRB1*11 (19.38%), DRB1*16 (13.95%), DRB1*03 (12.10%), DRB1*13 (10.99%) (table 2, figure 2).

**Table 2.** 6 other alleles revealed average frequencies, of 5% - 10% (DRB1*04, DRB1*07, DRB1*01, DRB1*15 and DRB1*14), while 2 were found in the 1%-5% frequency range and 3 had low frequencies, of less than 1% (DRB1*02, DRB1*05, DRB1*09) (table 2).

## DISCUSSIONS

The most frequent HLA-DRB1 alleles identified through the current research (frequencies >10%) were HLA-DRB1*16:01:01 (12.6%), HLA-DRB1*11:04:01 (12.1%), DRB1*03:01:01 (12%). The other more common variants detected (frequencies 5% - 10%) were HLA-DRB1*07:01:01 (9.3%), HLA-DRB1*01:01:01 (7.3%) and HLA-DRB1*11:01:01 (6.3%) and HLA-DRB1*13:01:01 (6%), while HLA-DRB1*15:01:01 was observed in approx. 4.4% of all tested individuals and HLA-DRB1*13:02:01 and HLA-DRB1*04:01:01 being at approximately a 3% frequency.

When adding the frequencies of all the variants for each HLA-DRB1 allele, the most common alleles are DRB1*11 (19.38%), DRB1*16 (13.95%), DRB1*03 (12.10%), DRB1*13 (10.99%), DRB1*04 (9.51%), DRB1*07 (9.26%), DRB1*01 (8.40%), DRB1*15 (5.93%), DRB1*14 (5.31%), while the other 5 detected HLA-DRB1 alleles (DRB1*10, DRB1*12, DRB1*02, DRB1*05, DRB1*09) had low combined frequencies (1.73% or lower).

In Europe, on average, the most common HLA-DRB1 variants are HLA-DRB1*07:01, followed by the DRB1*03:01, DRB1*15:01, DRB1*01:01, DRB1*11:01, DRB1*13:01, DRB1*04:01 and DRB1*16:01 alleles (listed in the descending order of their frequencies) [7].

The frequency observed for the most common HLA-DRB1 allele identified in our analyzed Romanian cohort (HLA-DRB1*16:01:01, 12.6%), if confirmed through further studies on wider populations, would be the second highest detected in all populations worldwide, only Bulgarians reveal higher frequencies for this allele (AF 15.5%) [7, 14]. The next population group, in descending order of allele frequencies for this HLA-DRB1 variant, would be Polish (8%) and then certain Russian populations (Belgorod and Nizhny Novgorod Russians and Bashkortostan Bashkirs) (AF 3.3% - 4.9%), but with much lower percentages for this allele [7].

Similarly, to HLA-DRB1*16:01:01, the frequency detected for the second most common allele in our cohort (HLA-DRB1*11:04:01, 12.1%), would also, if confirmed, be the second highest worldwide, being surpassed, again, only by the Bulgarian population (AF 15.5%) [7, 20]. The next high frequencies can be observed in the Paraguayan/Argentinian Guarani population (AF 10%) and different Russian populations (Russians from the Belgorod and Vologda regions and Tatars from Bashkortostan) (AF 5.54% - 5.88%), but also the Portuguese Madeira population (5.5%) [7].

The other more common HLA-DRB1*11 variant, HLA-DRB1*11:01, was observed in only approx. 6.3% of all analyzed Romanian individuals, almost half the frequency of HLA-DRB1*11:04. Similar findings were observed in continental and Crete island Greeks, Bulgarians, Macedonians, Croatians, Turks, and Italians, where HLA-DRB1*11:04 predominated over HLA-DRB1*11:01 [7, 21-30]. In contrast, the two alleles displayed equal or opposite frequencies in Central and Western European

countries, where the HLA-DRB1*11:01 allele is widely more prevalent [7].

Both the HLA-DRB1*16:01:01 and the HLA-DRB1*11:04:01 variants appear to be more specific for the populations belonging to or historically tracing back from the Eastern European, Caucasus, Black Sea and Balkan regions.

HLA-DRB1*16:01:01 is the most frequent in Macedonia (AF 14.9%), the second most prevalent in Bulgaria (after HLA-DRB1*11:04:01), Kosovo (12.9%) and Greece (HLA-DRB1*16:01, 4 digits, AFs 7.8% – 13.7%), the fourth in Slovenia, while the HLA-DRB1*16:01 allele (low resolution) is the third most frequent is among the population on the Croatian island of Krk (AF 11.2%) (and the HLA-DRB1*16 has the second highest prevalence with an AF of 11.8%). This is also possible in high percentages in Albania, where the second most commonly detected (low frequency) HLA-DRB1 variant is HLA-DRB1*16 (AF 12.4%), as is in Kosovo (AF 13.75%) [7, 21-30].

The HLA-DRB1*11:04:01 is possibly an even more ancient populational indicator, being observed in high frequencies in cohorts from Macedonia (second most frequent allele, AF 13.9%), Bulgaria (most common variant), Greece (HLA-DRB1*11:04 AFs 13.9% – 19.3%), Turkey (the most frequent HLA-DRB1 allele), also on the Croatian island of Krk (13.2%), Israeli Jews of Georgian descent (24.2%) and Polish Jews (17.8%), Armenia's combined Regions (11.5%), in Lebanon's Kafar Zubian (23.7%) and Niha el Shouff (17%) (where the DRB1*11:04 allele is the highest prevalent), Israeli Jews of Libyan descent (17.4%), Polish (17.8%) and Moroccan Jews (16.4%), or in Kosovo (10.5%), (HLA-DRB1*11:04 the second most common), parts of Black Sea Russia, Middle Eastern regions, and including Spanish Basque areas of Cantabria and Rome in Italy (the most prevalent variant) [7, 21-30].

A difference, nevertheless, can be observed between Eastern European countries (where both of these alleles are highly common) and the other above mentioned populations, (as are the Krk Island Croatians, the Armenians, the Spanish Cantabrian Basques and the Italians), where only HLA-DRB1*11:04:01 (or HLA-DRB1*11:04, depending on resolution) is the top or the second allele, but the HLA-DRB1*16:01:01 is one of the least frequent variants detected.

Both these similarities and differences could find an explanation in the historical genetic lineage of the peoples living in these areas, most of them being connected to the Pelasgians/Thracians/Getae/Dacians populations originating from these geographical areas (Turkey, Black Sea countries, Romanian regions, Bulgaria, parts of Albania and Macedonia, Kosovo, etc.). Also, the presence of the HLA–DRB1*11:04:01 allele in certain populations only (Basques, Armenians, Italians) can be explained by common ancient ancestors (Pelasgians/Thracians/Etruscans) of these peoples with the populations from the Eastern Europe and Balkan areas, the HLA-DRB1*16:01:01 variant appearing to be a mutational event which occurred after the separation of these groups, with a high specificity for Eastern Europe/Balkans.

Having reviewed related work, the frequencies of HLA-DRB1*16:01:01 and HLA-DRB1*11:04:01 were at a very high frequency compared to the prevalence in most Central and Western European populations.

In research articles from this European geographical area, and in the Romanian population as resulted from previous studies, the most common HLA-DRB1 variants are HLA-DRB1*03:01:01 and HLA-DRB1*07:01:01 [7,18,21-25]. The frequencies of the HLA-DRB1*07:01:01 and HLA-DRB1*03:01:01 alleles in the tested cohort were, by contrast, lower than those found in all populations from Central and Western Europe and also in past Romanian studies.

The third most common HLA-DRB1 variant in our studied population, HLA-DRB1*03:01:01 (11.98%), is one of the frequently identified alleles in various populations globally. Similar frequencies have been observed in populations such as USA Caucasians (12.47%), Portuguese Madeirans (12.2%), Israel Yemenite Jews (12%), Morocco Settat Chaouya (11.7%) and Atlantic Coast Chaouya (11.6%), Algerian Oran (11.6%), American citizens of Italian descent (11.2%) and Polish populations (10.9%). The highest frequencies worldwide for this variant can be seen in the Morocco Nador Metalsa (20.2%), Spanish Canary Islands (Gran Canaria island) (16.3%) and Northern Ireland (15.4%) populations [7].

Apart from the more frequently detected alleles in other populations throughout the globe, the current study also revealed the presence of 4 rare alleles (described in a maximum of 3 cohorts in published research results: HLA-DRB1*1:03:01 – 1 previous report; HLA-DRB1*11:01:02 – 2 reports; HLA-DRB1*04:08:01 – 2 reports; HLA-DRB1*13:0:01 – 3 reports) and also one new variant, not previously described in the literature (HLA-DRB1*04:06:02).

Further studies on larger Romanian cohorts need to be undertaken as to confirm these results and to understand their clinical, epidemiological, diagnostic, therapeutic or molecular implications.

## CONCLUSIONS

The current research provides important data on the HLA-DRB1 alleles frequencies among the Romanian population.

As expected and observed in previous studies, there are similarities and disparities when it comes to the more common and less frequent alleles between our analyzed cohort and the various European and non-European populations. Important similarities can be observed with different populations from Eastern Europe, the Balkans and Caucasus regions, possibly indicating a common genetic ancestry (documented by archaeological and historical data), while the Central and Western European countries show a constantly different constellation of HLA-DRB1 variants.

Further studies are needed for confirming and strengthening the findings of this research for the Romanian population.

## DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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