Finnish Red Cross Blood Service

and Doctoral Programme in Clinical Research,

University of Helsinki

Finland

ASPECTS OF HLA IN HEMATOLOGICAL STEM CELL TRANSPLANTATION

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DOCTORAL DISSERTATION

To be presented for public discussion with the permission of the Faculty of Medicine, University of Helsinki, in auditorium 1 of Metsätalo, Unioninkatu 40, Helsinki, on May 15th 2020, at 10 AM.

Helsinki 2020

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ISBN 978-952-5457-50-6 (print)

ISBN 978-952-5457-51-3 (pdf)

ISSN 1236-0341

http://ethesis.helsinki.fi

Unigrafia

Helsinki 2020

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Learn to say ‘I don’t know’. If used when appropriate, it will be often.

Donald Rumsfeld, 2001

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1 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I. Linjama T, Impola U, Niittyvuopio R, Kuittinen O, Kaare A, Rimpiläinen J, Volin L, Peräsaari J, Jaatinen T, Lauronen J, Saarinen T, Juvonen E, Partanen J, Koskela S. Conflicting HLA assignment by three different typing methods due to the apparent loss of heterozygosity in the MHC region. HLA. 2016 May;87(5):350-5

II. Linjama T, Eberhard HP, Peräsaari J, Müller C, Korhonen M. A European HLA Isolate and Its Implications for Hematopoietic Stem Cell Transplant Donor Procurement. Biol Blood Marrow Transplant. 2018 Mar;24(3):587-593

III. Linjama T, Räther C, Ritari J, Peräsaari J, Eberhard HP, Korhonen M*, Koskela S*. Extended HLA Haplotypes and Their Impact on DPB1 Matching of Unrelated Hematologic Stem Cell Transplant Donors. Biol Blood Marrow Transplant. 2019 Oct;25(10):1956-1964

IV. Linjama T, Niittyvuopio R, Tuimala J, Pyörälä M, Rintala H, Rimpiläinen J, Kauppila M, Peräsaari J, Juvonen E. Platelet donor selection for HLA-immunised patients; the impact of donor-specific HLA antibody levels. Transfus Med. 2017 Oct;27 Suppl 5:375-383

*Authors with an equal contribution on the article.

2 ABBREVIATIONS

ACI	Absolute post-transfusion count increment
aGvHD	Acute graft versus host disease
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BMDW	Bone Marrow Donors Worldwide
CML	Chronic myelogenous leukemia
CMV	Cytomegalovirus
CN-LOH	Copy number neutral loss of heterozygosity
DIC	Disseminated intravascular coagulopathy
DSA	Donor specific antibody
EBMT	European Society for Blood and Marrow Transplantation
EM	Estimation-maximization
FER	Finnish enriched rare
FRCBS	Finnish Red Cross Blood Service
FSCR	Finnish Stem Cell Registry
GvHD	Graft versus host disease
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
LD	Linkage disequilibrium
LOH	Loss of heterozygosity
Mb	Megabase
MDS	Myelodysplastic syndrome
MFI	Mean fluorescence intensity

NGS	Next generation sequencing
NMDP	National Marrow Donor Program
PCR	Polymerase chain reaction
PRA	Panel reactive antibody
RIC	Reduced intensity conditioning
ROC	Receiver operating characteristic
SSOP	Sequence specific oligonucleotide probe
SSP	Sequence specific primer
TC	Transplant center
URD	Unrelated donor
WMDA	World Marrow Donors Association
ZKRD	Zentrale Knochenmarkspender-Register, the German National Bone Marrow Donor Registry

3 ABSTRACT

Hematological stem cell transplantation (HSCT) is a widely used treatment for several life-threatening diseases, such as hematologic malignancies, hemoglobinopathies and severe immunodeficiencies. HSCT can be either autologous (the patient's own hematological stem cells are collected and later returned) or allogeneic (transplanted stem cells are collected from another individual). Since the description of the Human Leukocyte Antigen (HLA) system in the late 1950's, it has become evident that for a successful allogeneic HSCT, a sufficient level of HLA matching between the patient and the stem cell donor must exist.

A HSCT for an individual patient includes numerous steps, and HLA laboratories as well as stem cell donor registries play an important role in many of these. For a successful allogeneic HSCT donor search, the first prerequisite is an accurate HLA typing of the patient and the potential allogeneic donors; for the patients with no HLA identical siblings, an adequate number of unrelated registry donors is beneficial; even post-transplant an HLA laboratory is sometimes needed, as all HSCT patients require blood product support and this concerns also patients who are sufficiently HLA alloimmunised to be refractory for standard platelet products. This thesis aims to answer HLA related questions that have arisen from the everyday work of an HLA laboratory and stem cell registry.

HLA typing by the current DNA based methodologies in an accredited experienced HLA laboratory is usually a straightforward process. However, as acute leukemias are a frequent indication for HSCT, HLA typing is often performed from samples containing a large proportion of leukemic cells, which are sometimes known to carry loss of heterozygosity (LOH) of the HLA complex. LOH may cause a falsely homozygous typing result for one or several of the HLA genes in the same haplotype. We described the HLA typing history of five patients with LOH, and compared different HLA typing methods to assess their abilities to detect both HLA haplotypes

of LOH patients. We further performed a retrospective typing of an independent study set of hematologic patients with at least two adjacent homozygous HLA loci, but no erroneously reported homozygous results were detected.

To assess the utility of maintaining a Finnish unrelated donor stem cell registry, and to facilitate the predictability of Finnish low resolution typed registry donors' matching grade for individual patients, we calculated the Finnish low and high resolution HLA haplotype frequencies. This was performed by using the HLA typing results or Finnish Stem Cell Registry (FSCR) members. The acquired frequencies were compared to the similarly calculated frequencies of Germans, Swedes and Russians. The results show that 25 % of Finns carry HLA haplotypes that are heavily enriched in the Finnish population and rare among other Europeans. Finnish patients carrying these haplotypes are more likely to receive domestic or mismatched stem cell transplants. The results indicate that there is a special value in maintaining a Finnish Stem Cell Registry, regardless the high numbers of members in the much larger European registries.

HLA-DPB1 genes were long considered unimportant in the allogeneic HSCT context, but it was since shown that there are several instances where either DPB1 matching or at least avoidance of certain mismatches is beneficial. Finnish extended 6 locus HLA haplotypes were calculated using the DPB1 typed FSCR donors as the study population. The DPB1 associations of different 5-locus HLA haplotypes were widely divergent. The associations seem to be at least partly specific for Finland, as the retrospectively studied transplant pairs were significantly more often DPB1 matched if the donor had been domestic. International donor searches for Finnish patients, carrying different combinations of 5-locus HLA-haplotypes, resulted in diverse proportions of DPB1 matched donors, showing that non-random DPB1 associations are likely to exist in other populations as well. The results indicate that population specific extended HLA haplotypes can be assessed and might be used in predicting the probability of undesirable DPB1 mismatches in registry donor searches.

Abstract

In the first weeks post-HSCT all patients, even those with optimally matched transplants, undergo a period of severe cytopenias, and require platelet transfusion support. Patients with pre-formed HLA-antibodies are often refractory to standard platelet products, and need platelets that are collected from specifically HLA selected blood donors. There are however several different selection methods, and no consensus as to which of the methods is best as to the platelet increments, let alone the clinical efficacy on bleeding and mortality. The impact of different levels of platelet donor specific HLA antibodies of the patient on the platelet transfusion increments was analyzed in a Finnish sample of HLA immunized platelet transfusion refractory patients. Donor specific antibody levels higher than 1000 MFI (Mean fluorescence intensity) were shown to be an independent risk factor for inferior transfusion responses, whereas a limited number of structural differences (depicted by the number of eplet mismatches by the Matchmaker algorithm) between the patient's and donor's HLA antigens was not a sufficient predictor of HLA antibody levels in our group of highly alloimmunised patients.

4 INTRODUCTION

Hematological stem cell transplantation is an established treatment for several life-threatening diseases, and more than 18 000 allogeneic transplants were performed in 2017 in Europe alone ((Passweg *et al.*, 2019). The first successful HSCTs were performed from the patients' HLA identical siblings. However, since 1980's, availability of unrelated donors (URD) has vastly increased and the treatment modalities improved so that today, the outcomes of optimally HLA matched unrelated donor HSCTs are often considered comparable to transplants from HLA identical siblings. In Europe in 2017, 32 % of allogeneic transplants were performed from the patient's HLA identical sibling, 16 % from an HLA mismatched related donor and 50 % from an unrelated donor, provided by the worldwide network of unrelated donor registries; 2 % of transplants were supplied by cord blood banks (Passweg *et al.*, 2019). In Finland in 2018 131 allogeneic HSCTs were performed. 85 (65 %) of them were with an unrelated donor, 22 (17 %) with an HLA identical sibling, 22 (17 %) with an HLA mismatched family donor and two (1.5 %) with a cord blood unit.

Although the Human Leukocyte Antigen (HLA) –system was first discovered 60 years ago and has since been actively studied, new un-answered questions arise yet today, due to the system's immense variability and its central role in both adaptive immunity and transplantation immunology. The gradual development of histocompatibility testing was a necessary prerequisite for successful allogeneic transplantation, and it has vastly improved in accuracy, affordability and work flow since it was first developed in the late 1950's. For several decades the bulk of histocompatibility testing was performed by serological methods, which were accurate enough for establishing HLA identity between siblings in a family. However, the widespread use of DNA based methods around the turn of the millennium was required before transplantation outcomes with unrelated donors became comparable

with sibling transplants. The DNA based methods, specifically sequence based typing (SBT) methods, allow accurate donor and patient HLA matching at the level of single nucleotide polymorphisms of classical HLA molecules. Subsequently, the criteria for "optimal" HLA matching between the patient and the donor have become more stringent, requiring much larger numbers of volunteer unrelated donors in the worldwide registries than was originally thought necessary.

In Finland, histocompatibility testing required in solid organ and hematological stem cell transplantations is exclusively performed by the Finnish Red Cross Blood Service (FRCBS) histocompatibility laboratory. Thus, it is relatively large in the European setting and usually able to introduce new analysis technologies without undue delay. The Finnish Stem Cell Registry also belongs to the FRCBS, enabling a very close collaboration between the laboratory and the stem cell registry. Since all HLA selected platelets in Finland are also supplied by the FRCBS, the same HLA specialists are able to engage with all the diverse aspects of histocompatibility encountered during the hematological transplant process. Because of the size of the laboratory and the versatility of the HLA consultants' tasks, also diverse questions concerning optimal practices arise during the daily work. Many of these have not yet been studied either because of relatively new laboratory methods or sometimes national idiosyncrasies. The studies making up this thesis have been done to answer such questions. They have thus the very pragmatic benefit of being readily translatable to the everyday practices of HLA consultants serving blood banks, HLA laboratories and stem cell registries.

5 REVIEW OF THE LITERATURE

5.1 HLA

5.1.1 Basics of HLA

The Human Leukocyte Antigen –system is the human counterpart to the Major Histocompatibility Complex, a set of molecules expressed in all vertebrates. The protein products of these genes are expressed in hundreds of thousands of copies on all human cells with the exception of erythrocytes. They are essential for the normal function of T lymphocytes, as indicated by the recurrent severe infections seen in the rare individuals with a deficiency of either class I or class II HLA molecule expression (Hanna and Etzioni, 2014).

In humans, the HLA gene complex is located in the short arm of chromosome 6 (6p21.3). Significant linkage disequilibrium (LD; alleles at different loci in nonrandom association with each other (Slatkin, 2008)) is known to span this area. Hence, the HLA genes are usually inherited from the parent to the offspring as one block, called the haplotype; one HLA haplotype from each parent (Figure 1).

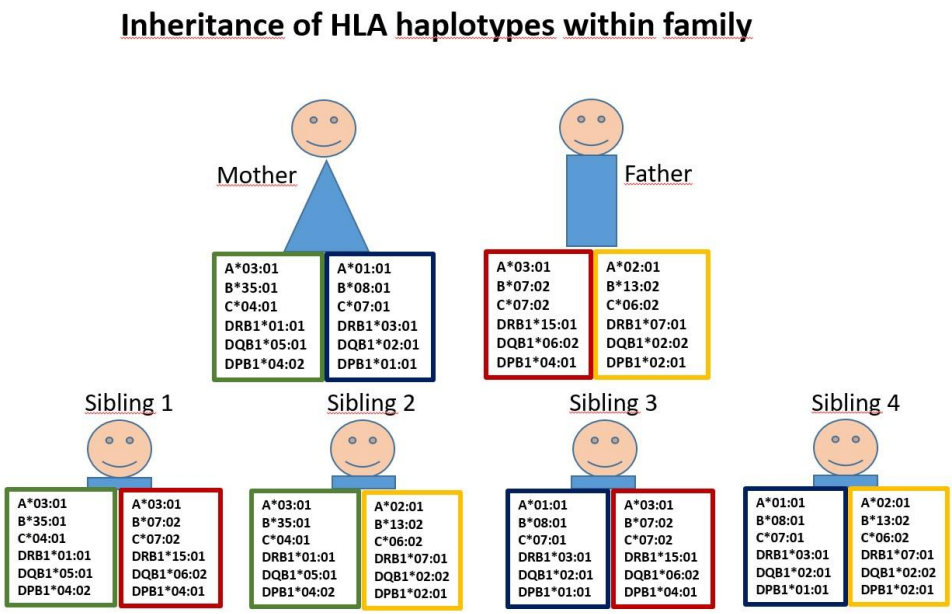

Figure 1. *Each offspring inherits one HLA haplotype from each parent. For any two siblings, there is a 25 % probability of carrying the same two parental HLA haplotypes, a 50 % probability of sharing one haplotype in common, and a 25 % probability of sharing no HLA haplotypes.*

HLA genes are the most polymorphic ones in the human genome, with almost 24 000 alleles and more than 15 000 proteins known today (*https://wwwdev.ebi.ac.uk/ipd/imgt/hla/stats.html*, 2019).

HLA-A, *HLA-B*, *HLA-C*, *HLA-DRB1*, *HLA-DQB1* and *HLA-DPB1* are the classical HLA genes. Of these, the three first ones belong to HLA class I and share a similar structure at gene and molecular level. The rest of the genes belong to HLA class II, and their structure differs from that of Class I molecules. The structure of HLA molecules (both class I and class II) is depicted in Figure 2. Class I molecules are heterodimers consisting of a heavy chain (often called α chain), coded by the HLA-A,

-B, or -C gene, and a light chain, β_2 microglobulin, coded by *B2M* gene in chromosome 15 (P J Bjorkman *et al.*, 1987; Klein and Sato, 2000). The heavy chains have a specific structure in which different domains of the proteins are encoded by separate exons. A leader peptide is encoded by exon 1. Exons 2 and 3 encode two of the three cell surface domains, α_1 and α_2, which are the most variable parts of the molecule. Together α_1 and α_2 form the highly variable peptide binding groove of the molecule. Domain α_3 is less variable than α_1 and α_2 and is coded by exon 4 of the HLA Class I gene. The transmembrane part of the molecule is coded by exon 5, the cytoplasmic tail by exons 6 and 7, and the 3' untranslated region (UTR) by exon 8 (Malissen, Malissen and Jordan, 1982). The β2m chain has no transmembrane or cytoplasmic part and is non-covalently bound to α_3 domain of the heavy chain (P J Bjorkman *et al.*, 1987).

The structure of class II molecules differs from class I molecules: they also consist of two chains, α and β, but these are both coded by HLA-D genes, (letters A and B in the name of the respective gene denoting whether the gene codes for the α or for the β chain), and both chains of the class II HLA molecules carry a peptide binding domain (α_1 and β_1, respectively), which together form the peptide binding groove (Brown *et al.*, 1993; Klein and Sato, 2000). As in class I genes, exon 1 codes a leader peptide, while the peptide binding domains of class II molecules are coded by exon 2 of the respective HLA-D genes. The α_2 and β_2 domains are coded by exon 3, transmembrane anchor and cytoplasmic tail by exons 4-5 and 3'UTR by exon 6.

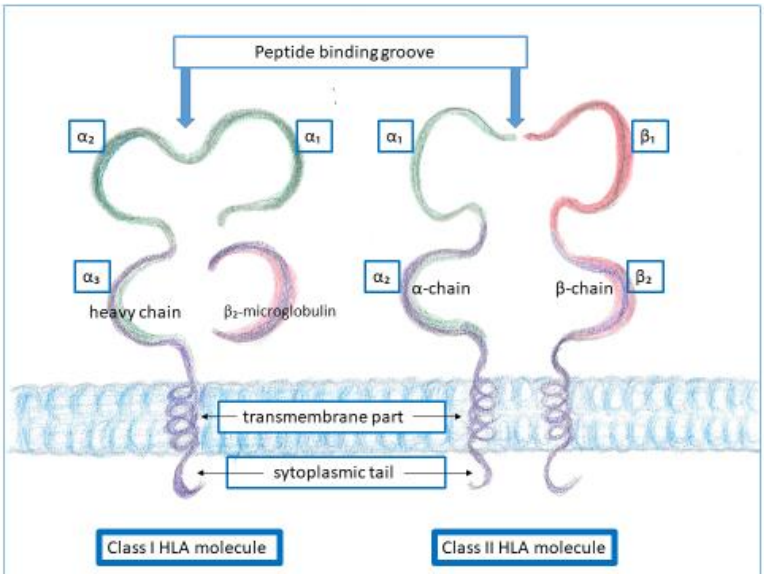

Figure 2. *Structure of class I and class II HLA molecules. The most variable parts surround the peptide binding groove: α_1 and α_2 domains of the class I molecule and the α_1 and β_1 domains of the class II molecule.*

The expression of HLA molecules is co-dominant, i.e. the HLA molecules encoded by the HLA alleles inherited from each parent are both expressed on the cell surface. HLA Class I molecules are expressed on all nucleated cells, but Class II molecules normally only on cells that are specialized in antigen presentation, such as B-lymphocytes, dendritic cells and cells of the monocyte-macrophage-lineage.

The function of the HLA molecules is to carry and present short peptides to T-lymphocytes and thus continuously help them control the antigen content of the cells. The HLA- peptide complex also plays a central role in the development of immunological tolerance and the selection of T-cell receptor repertoire in the thymus

(Partanen, 2003). The inherited structure of the peptide binding groove determines the affinity, size and repertoire of the different peptides that are bound and presented by different HLA molecules (P.J. Bjorkman *et al.*, 1987). Class I peptides present peptides that originate within the cell: cell's own degraded proteins and proteins that are produced because of a viral infection, and the basic function of them can thus be described as targeting the cytotoxic immune activation against cells that synthesize foreign structures, as in connection with viral infection or cancer (Pettersson, Partanen and Vakkila, 2007). Class II HLA molecules present peptides that have been endocytosed from the outside of the cell, such as peptides originating from bacterial infections. The receptors of T helper cells (CD4-positive cells) recognize and are activated by class II HLA molecule-peptide complexes, and cytotoxic or killer T-cells (CD8 positive T-cells) by class I HLA molecule-peptide complexes. Interaction between the HLA molecule-peptide complexes and T-cells via T-cell receptors is necessary for the activation and normal function of T-lymphocytes. (Burmester and Pezzutto, 2003).

5.1.2 Population genetics of HLA

The HLA complex is the most variable region of the human genome, and there is strong evidence that the diversity of HLA alleles is maintained by balancing selection, the kind of selection that results in increased genetic diversity (Brandt *et al.*, 2018). The HLA allele distribution varies between different populations (Schipper *et al.*, 1997) although most of the variation is between individuals, and a lower proportion between populations and continents: for example, for the A locus the variance was reported to be 88.5 % between individuals, and 6.2 % and 5.3 % between populations and continents respectively (Sanchez-Mazas, 2007). Worldwide, the diversity of HLA alleles is primarily correlated to the population's distance from east Africa (Prugnolle *et al.*, 2005), thus diminishing for example in Europe from southeast to northwest. As the primary function of HLA alleles is to present pathogen derived peptides to the T lymphocytes, part of the diversity has also been speculated to result from pathogen derived selection, and it has been shown that after adjusting for the

effect of human migration history, HLA Class I allele diversity is larger in areas with the largest diversity of different pathogens, especially viruses (Prugnolle *et al.*, 2005).

HLA genes are located in an approximately 3,6 Mb (Megabase) segment (Trowsdale, Ragoussis and Campbell, 1991) in the p arm of chromosome 6, and this block, the HLA complex, is characterized by a strong linkage disequilibrium resulting in long-range HLA haplotypes. In contrast with the balancing selection for individual HLA alleles, the HLA haplotypes seem to be affected by purifying selection (Alter *et al.*, 2017). As a result, the highly polymorphic HLA alleles are not inherited in random combinations, but usually *en bloc* from one generation to the next (Figure 1). The HLA haplotype frequencies seem to be more population specific than individual alleles. Of the 64856 5-locus haplotypes reported by the US National Marrow Donor Program NMDP (Gragert *et al.*, 2013), only 10 018 have a reported frequency among European Caucasians and 1350 among donors of Japanese ancestry, and there are actually only 188 haplotypes that have a frequency among both European and Japanese populations. Likewise, although Asian, Caucasian and African American populations individually are all reported to have approximately 10 000 haplotypes with known frequencies, of these, only 574 have a frequency in all three broad ethnic categories. Even at the level of geographic parts of Germany, significant haplotype diversity differences have been found (Schmidt *et al.*, 2010).

The sparse population and relative isolation of Finns in the North-Eastern corner of Europe has led to a concept of "Finnish Disease Heritage", meaning a group of mostly recessively inherited diseases that are strongly overrepresented in Finland (Norio, 2008). It is conceivable that the same background might lead to uniquely enriched HLA haplotypes as well. Accordingly, previous studies on Finnish HLA characteristics have consistently reported significant differences from other European born populations (Lokki and Julin, 1982; Sirén *et al.*, 1996; Haimila *et al.*, 2013), even though the typing resolution has been low in the first two studies and the number of HLA typed individuals relatively small in the last one.

The differences of HLA haplotypes and haplotype frequencies between different populations have a profound effect on unrelated donor procurement: as donors with European ancestry are over-represented in the worldwide stem cell donor registries, patients of other ethnicities have much lower probabilities of finding optimally HLA matched stem cell donors (Pidala *et al.*, 2013; Gragert *et al.*, 2014).

5.2 HLA TYPING METHODS

5.2.1 Serology

The HLA system was first recognized in the 1950's by using sera of multiparous women who had been alloimmunised by pregnancies. These sera agglutinated the white blood cells of their husbands, and varying proportions of other individuals (Jager, Brand and Claas, 2019). The most widely used serological typing method, complement mediated microcytotoxicity assay, was developed based on this finding, and even today serology is the only routine typing method that recognizes the actual antigens expressed on the cell surface.

5.2.2 DNA based methods

Serology remained the primary typing method until the 1990's (Woszczek *et al.*, 1997), but the vast variability of the HLA system has only gradually been exposed by the development of DNA based methods. Although these were available already in the 1970's after the advent of the recombinant DNA technology, they became more widely applicable only after the polymerase chain reaction (PCR) was introduced in 1985 (Erlich, 2012).

The three most widely used DNA based typing methods have been SSP-PCR (sequence specific primer-PCR), SSOP (sequence specific oligonucleotide probes) and DNA sequencing.

5.2.3 Sequence Specific Primers (SSP-PCR)

In the SSP-PCR method, isolated genomic DNA is amplified with a set of oligonucleotide primers that are complementary to one to several specific HLA alleles. An amplification product is only produced if any single primer pair is complementary to the DNA that is being analyzed (Chandraker *et al.*, 2012). SSP assay is limited by requiring an increasing number of separate PCR reactions to achieve high resolution, and by not being suited for high volume automation.

5.2.4 Sequence specific oligonucleotide probes (SSOP)

In the SSOP technique, the PCR product of a HLA gene is let to hybridize with probes that are designed based on sequences of different HLA alleles. Only probes complementary to the DNA under analysis hybridize with the PCR product. In different SSOP applications, the probes can be immobilized in different ways: early on, on nylon membranes (Erlich, 2012), and later, on variably colored microbeads as in the Luminex technology. In Luminex, the SSOP technique combines microbeads and flow cytometry with automated analysis, allowing more high-throughput typing compared to SSP and earlier SSOP techniques (Heinemann, 2009). A known weakness of SSOP assays is that they provide similar hybridization and detection conditions for all probes. This results in potentially sub-optimal conditions for some of the probes, thus occasionally leading to falsely positive or negative fluorescent signals (Eng and Leffell, 2011).

5.2.5 Sanger sequencing

To detect all novel mutations in the HLA genes and to discern between all different alleles, it is necessary to determine the exact nucleotide sequence of the gene. This was made possible by the sequencing techniques, of which Sanger sequencing was introduced in 1977 and has for decades been the golden standard of high resolution

typing methods. It is based on synthesis of a complimentary DNA template for the single stranded DNA that is being analyzed; the synthesis is initiated by annealing oligonucleotide primers to the single stranded DNA and the extension of the synthesis is terminated at variable lengths by adding 2′,3′-dideoxynucleotides (ddNTPs) by DNA polymerase instead of the natural 2′-deoxynucleotides (dNTPs) (Sanger, Nicklen and Coulson, 1977; Metzker, 2005). Even though Sanger sequence analysis became more automated by the development of fluorescent labels, capillary electrophoresis, laser techniques and software, it is still relatively slow and laborious and so not optimally suited for high throughput HLA typing. Also, with the constant increase in the number of known HLA alleles, the exclusion of HLA allele ambiguities with Sanger sequencing alone has become a moving target.

5.2.6 Next Generation sequencing (NGS)

The first NGS platform was introduced in 2005 (Margulies *et al.*, 2005), and these techniques are now replacing Sanger sequencing at least in high throughput HLA laboratories. The biggest difference to the Sanger methodology is that large numbers of clonal sequencing reactions can be performed in parallel, diminishing the required hands-on time and dropping the cost of sequencing. The variability of the HLA system has been a challenge even for the NGS platforms, and they require sophisticated bioinformatics tools (Gabriel *et al.*, 2014). The clonality of the NGS sequencing products currently diminishes the number of high resolution level ambiguities close to zero, and NGS also makes it possible to sequence whole genes, thus including all exons and introns in the final typing result. Specifically, NGS has revolutionized the upfront HLA typing of new registry donors, enabling faster and more reliable donor selection for patients in need of an unrelated stem cell donor. However, even NGS shares a limitation common to all DNA based assays: dependency on the quality of primer design. In all assays where both alleles are amplified simultaneously, sub-optimal primers may lead to amplification imbalance (Juha Peräsaari, FRCBS histocompatibility laboratory, personal communication). Unspecific primers on the other hand may amplify background fragments not

belonging to the target locus (Mann *et al.*, 2009). A critical check-up of primary analysis data is thus always necessary if the typing result is homozygous or contains rare or unexpected alleles. It is also important that the laboratory has access to more than one typing assay to resolve problematic situations.

5.3 LOSS OF HETEROZYGOSITY

In human malignancies, loss of heterozygosity (LOH) is a common form of genetic abnormality (Zheng *et al.*, 2005). It is known to favor the development of a malignant transformation by the loss of normally functioning tumor suppressor genes (Couto, 2011; Ryland *et al.*, 2015), or by duplicating the oncogenic mutations while losing the remaining normal allele (O'Keefe, McDevitt and Maciejewski, 2010). LOH may also assist the survival of malignant cells by downregulating HLA Class I expression, thus helping them escape T-cell immune surveillance (McEvoy, Morley and Firgaira, 2003). LOH can lead to a loss of an entire chromosome or chromosomal region copy number, or it can be copy number neutral (CN-LOH). In CN-LOH the remaining chromosome region, either maternal or paternal, is duplicated (uniparental disomy, UPD), making CN-LOH undetectable by conventional karyotype analysis (O'Keefe, McDevitt and Maciejewski, 2010). In myeloid malignancies, copy-neutral-LOH is known to exhibit a non-random distribution: the most frequently involved chromosomes are 4, 7, 13 , 11 and 17, but there are also regions like 5q and 20q that are almost never affected (Xu *et al.*, 2018). Although the HLA region in chromosome 6 is thus not the most frequent of the involved regions, several reports of LOH impacting the initial HLA genotyping of individual leukemia patients have been published (Park *et al.*, 2011; Dubois *et al.*, 2012; Lobashevsky *et al.*, 2019).

5.4 HEMATOLOGICAL STEM CELL TRANSPLANTATION

The idea of HSCT is to reconstitute the missing or deficient hematopoietic cell lineages with normal cells, which are capable of continuous self-renewal. In addition, the transplanted immune cells provide anti-tumor effect when the transplant is performed for a hematological malignancy.

5.4.1 HSCT immunology

The human immune system has two major arms: the innate and the adaptive. The determining characteristics of the adaptive immune system are the cells' capability of specific recognition of a foreign antigen, clonal proliferation of the effector cells that encounter their specific antigen, and memory, which enhances the expansion of the effector cells when the antigen is met and recognized repeatedly (Petranyi, 2002). These properties are based on a system of highly variable receptors on the effector cells of the adaptive immune system, T and B lymphocytes (Burmester and Pezzutto, 2003).

Alloreactivity is based on the recognition of alloantigens (peptides derived from cells of a different individual of the same species) by T-cells. This can happen via three different pathways: direct, indirect or semi-direct (Marino, Paster and Benichou, 2016). In the direct pathway, the effector T-cells recognize intact foreign HLA molecule-peptide-complexes on the allogeneic antigen presenting cells (APCs); in the indirect pathway, the effector cells recognize processed allogeneic HLA molecules on self-APCs (Game and Lechler, 2002). The term semi-direct pathway depicts situations in which HLA class I and class II molecules of the recipient and donor are expressed on each other's dendritic cells as the result of transfer or "cross-dressing" (Marino, Paster and Benichou, 2016).

As to alloreactivity, HSCT is a unique treatment modality: the immune systems of both the recipient and the donor are able to recognize the other as non-self, and initiate an attack on each other's cells. The recipient's immune system may prevent the engraftment of the donor's transplanted cells (non-engraftment), or reject them

later, after initial engraftment. This is not a common problem in HLA identical sibling or matched URD transplants, but a well-recognized risk in HLA mismatched transplants. However, even in HLA compatible HSCT, a much more frequent complication is Graft versus Host Disease (GvHD), in which the donor's T-cells attack the patient's cells, causing damage in the target tissues (Welniak, Blazar and Murphy, 2007). As GvHD was apparent in 35 % of HLA identical sibling transplants and 41 % of HLA compatible URD transplants (Spierings *et al.*, 2013), the attack is not dependent solely on HLA matching. In sibling transplants the risk increases with increased amount of mismatching in minor histocompatibility antigens (miHA), but in unrelated transplants much of the increase seems to be brought about by DPB1 mismatches (Martin *et al.*, 2017), as in unrelated transplants 10/10 matching is usually targeted, and internationally more than 80 % of 10/10 matched transplants are DPB1 mismatched (Lee *et al.*, 2007; Shaw *et al.*, 2007; Pidala *et al.*, 2014).

5.4.2 Development of HSCT

Interest in the possibility of replacing an individual's hematopoietic system by hematologic stem cell transplantation arose after the atomic bombings of World War II and the subsequent cold war, as it had become evident that the destruction of the victims' hematopoietic system was the usual cause of death after fatal radiation injury (Okita, 1975). The first attempts at HSCT in the 1950's and early 1960's were not successful, and all patients died; the causes of death were usually either relapse of the primary disease or immunological reactions (Singh and Mcguirk, 2016).

The first successful HSCTs were performed in the Netherlands and the USA in late 1960's. The donors were HLA identical siblings, and the transplants were performed in the pediatric setting, with the first transplant indication being severe combined immunodeficiency, SCID (Jager, Brand and Claas, 2019). In early 70's, also patients with severe aplastic anemia (SAA) and leukemias were transplanted, while only HLA identical siblings were considered acceptable donors. The pioneer of allogeneic

HSCT was Nobel Laureate Professor E. Donnall Thomas, who reported a series of a hundred allogeneic transplantations in 1977 (Thomas *et al.*, 1977). The first successful transplant from an unrelated donor was performed for leukemia in 1979 (Jurick *et al.*, 2016).

At first, stem cell transplants were only regarded as a replacement of the patient's own bone marrow, which either did not function normally, as in immunodeficiencies and aplastic anemias, or was destroyed by radiation and chemotherapy, as in leukemia treatment. However, early on there were indications that the transplanted cells might also have an immunologic effect, recognizing and destroying the patient's remaining malignant cells (Thomas *et al.*, 1977; Weiden *et al.*, 1979; Gale *et al.*, 1994). This is thought to be especially important concerning the malignant stem cells that are not actively proliferating and are thus more resistant to chemotherapy than other leukemic cells (Copelan, 2006).

5.4.3 Indications for allogeneic HSCT

Although the earliest successful allogenic hematological stem cell transplantations were performed for pediatric patients with severe immunodeficiencies and aplastic anemias, leukemias soon became the most frequent indication (Thomas *et al.*, 1977; Copelan, 2006). Early on, when the risk of transplant related mortality and graft rejection especially in unrelated donor transplants was significantly higher than today, chronic myelogenous leukemia (CML) was the most frequent malignant indication for HSCT, as no other effective treatment options for CML were available at the time. Acute leukemias were treated by HSCT in more advanced disease phases than today – at relapse or in second remission (Gajewski, Cecka and Champlin, 1990). The only established indications for URD HSCT twenty years ago were chronic phase CML, Philadelphia positive acute lymphoblastic leukemia (ALL) in first remission and other ALLs in later remissions. In addition, there were two optional indications: poor prognosis acute myeloid leukemia (AML) in remission and myelodysplastic syndrome (MDS), as reported by Madrigal (Madrigal *et al.*, 1997).

Approximately 70 % of patients (Thomas *et al.*, 1977) had no HLA identical sibling donor available, which left a large number of patients with other HSCT indications outside of this treatment modality. As the myeloablative conditioning that was required was toxic, only relatively young, otherwise healthy patients could be transplanted. Both these factors severely limited the use of HSCT. Consequently, the number of HSCTs in Europe in 1995 was 3804, 20 % of which were from an unrelated donor.

However, at this time, the number of unrelated transplants was rapidly increasing, the rise being 100 % from 1994 to 1995 (Gratwohl, Hermans and Baldomero, 1997; Madrigal *et al.*, 1997) and the number of both allogeneic transplants and the proportion of unrelated donors have subsequently both risen at a fast pace. In 2015, European Society for Blood and Marrow Transplantation (EBMT) reported more than 17 000 allogeneic transplants in Europe (48 countries) and 9 affiliated countries (all situated in Africa or the Middle East) alone. Of these, more than 50 % were from an unrelated donor (Passweg *et al.*, 2017).

Today, the most frequent indications for allogeneic transplantation are acute leukemias and myelodysplastic syndromes, together covering 67 % of indications in 2015. Less frequent indications are multiple myeloma (7 %), chronic leukemias (4 %), lymphomas (7 %), bone marrow failure (5 %), hemoglobinopathies (3 %) and different immune deficiencies (3 %) (Passweg *et al.*, 2017). As to the indications, the biggest change between the 20th and the 21st centuries has been the dramatic reduction of chronic myelogenous leukemia (CML) as a transplant indication, due to the introduction of tyrosine kinase inhibitors in the 1990's (Innes, Milojkovic and Apperley, 2016).

5.4.4 Conditioning and immunosuppression

Before the transplantation of stem cells from an allogeneic donor, the patient needs pre-transplant conditioning. There are several reasons for this: the allogeneic cells

must have sufficient space in the bone marrow to engraft; the patient's own immune system must be sufficiently weakened not to reject the transplanted cells, and in treatment of malignant diseases, the leukemia cells must be eradicated as profoundly as possible (Jurick *et al.*, 2016). Younger patients with no significant comorbidities can withstand myeloablative conditioning, which causes long-term three-lineage aplasia of the marrow and allows rapid engraftment of the transplanted cells. As leukemias are much more frequent in the elderly, also different reduced intensity conditioning (RIC) regimens have been developed to allow transplantation also for older age groups and patients with comorbidities. Compared to the myeloablative treatment, RIC-conditioning relies more heavily on the graft versus leukemia effect of the transplant (Joensuu *et al.*, 2006). Post-transplant, different immunosuppressive treatments are used to prevent GvHD and graft rejection (Carreras *et al.*, 2019).

5.4.5 Complications

The major clinical complications of HSCT are both acute and chronic graft versus host disease (GvHD), graft rejection and failure, treatment-related toxicity and various infections due to the ensuing prolonged immunodeficiency. For patients with a normal post-transplantation course, immunological function gradually normalizes, but until this happens, a heightened risk of different infectious complications prevails. (Carreras *et al.*, 2019).

5.4.6 Development of histocompatibility assessment in HSCT

The first, unsuccessful bone marrow transplants in the 1950's, were collected from cadavers (Singh and Mcguirk, 2016). These transplantations were performed without any attempt to HLA matching, as no HLA typing methods were yet in use (Jager, Brand and Claas, 2019). By the end of the 1960's however, the HLA system was already well known, and the histocompatibility of HLA identical siblings could be assessed by family typing with serological methods. As only 25 % of siblings are HLA identical with each other, most patients do not have an HLA identical donor in their immediate family. This was a serious limitation for HSCTs through the 1970's

and 1980's even for patients with a European background, who today have a high probability (Gragert *et al.*, 2014) of finding an optimally matched URD.

The first successful unrelated donor hematological stem cell transplant for leukemia was performed for a patient who, according to the reported HLA typing result (Hansen *et al.*, 1980) probably carried the two most frequent European HLA haplotypes. Because of this, an HLA-A, -B, -DRB1 matched donor was found from a very small number of HLA typed volunteers (Hansen *et al.*, 1980). From today's perspective, it is probable that the donor and the patient were actually fully matched for five classical HLA genes (*www.Haplostats.org*, 2019), due to the minimal allele variation in these conserved ancestral HLA haplotypes. Although the patient after two years died of leukemia relapse, this transplant with no signs of acute or chronic GvHD and rapid engraftment showed the feasibility of using well matched unrelated donors when no matched family donors were available.

Many of the first volunteer unrelated donors were recruited from blood donors, who had previously been HLA-A and HLA-B typed for platelet donation purposes (Mccullough *et al.*, 1986; Mcelligott, Menitove and Aster, 1986; *www.ZKRD.de*, 2019). Selective DRB1 typing could then be performed for donors who were putatively HLA-A and –B matched with the patient. Thus, in early 1990's only 30 % of registry donors in the Bone Marrow Donors Worldwide (BMDW) database were HLA-DRB1 typed (Buskard and Stroncek, 1993). The rest of the HLA genes were at the time considered unimportant, and it was unclear whether even split level matching of HLA-A and -B antigens or extended DR-typing would be beneficial (Gajewski, Cecka and Champlin, 1990). In 1993, mismatches of HLA-A or HLA-B antigens were deemed as "minor" if the respective antigens were serologically cross-reactive (Beatty *et al.*, 1993)

As knowledge on transplant outcomes increased and typing methods improved, recommendations began to change. By the end of the 1990's, the World Marrow

Donor Association (WMDA) recommended HLA-A and HLA-B typing at split antigen level and DRB1 typing both at the time of registry donor recruitment, and later at confirmatory typing of both patients and donors. DNA based typing methods had by then become more easily available, and allele level DRB1 typing was recommended (Hurley *et al.*, 1999). By the beginning of 2000's, the recommended typing resolution for identical sibling versus unrelated donor transplantations had become different: low resolution typing was considered sufficient in family transplants, but higher resolution typing was already recommended for URD transplants; HLA-C and HLA-DQB1 typing was considered useful for developing accurate search strategies (Hurley, Fernandez-Vina and Setterholm, 2003).

The significance of high resolution level matching and HLA-C matching became evident gradually in the early 2000's (Flomenberg *et al.*, 2004; E. W. Petersdorf *et al.*, 2007; Lee *et al.*, 2007; Fürst *et al.*, 2013). Today, the HLA match degrees that are most widely considered as optimal are an 8/8 or a 10/10 match. The former means that the patient and donor are matched at high resolution level for HLA-A, -B, -C and -DRB1 genes (high expression genes), and the latter requires a match also at HLA-DQB1, which is a lower expression HLA gene together with HLA-DPB1 and HLA-DRB3-5. Matching requirements for the low expression HLA genes is more controversial than of the high expression genes (Fernandez-Viña *et al.*, 2013).

5.4.7 Impact of HLA matching in allogeneic HSCT

Knowledge of the required HLA matching degree for optimal transplant outcomes has gradually increased over decades. In 1990's when the unrelated donors were only phenotypically HLA-A-B-DRB1 matched, registry donor transplants were associated with significantly higher morbidity and mortality rates, due mostly to aGvHD (acute graft versus host disease) but also because of a higher risk of transplant rejection and susceptibility to infections (J. Madrigal *et al.*, 1997). Today, HLA matching between the patient and the URD is considered as the one most important donor-related factor

predicting the outcome of the transplant, and transplant outcomes with matched unrelated donors have become comparable with sibling donors (Ho *et al.*, 2011; Robin *et al.*, 2013; Dufour *et al.*, 2015). A single antigen or allele level HLA mismatch at the HLA-A, -B, -C or -DRB1 gene has repeatedly been shown to be associated with an approximately 8-10 % lower probability of overall survival, and also with a higher risk of both acute and chronic GvHD and transplant related mortality. For DQB1 mismatches, most of the same studies have not shown an impact on overall survival at all, if the donor is 8/8 matched with the patient (Flomenberg *et al.*, 2004; Lee *et al.*, 2007; Fernandez-Viña *et al.*, 2013; Pidala *et al.*, 2014), or the slightly higher mortality risk has been associated with antigen level mismatches only (Fürst *et al.*, 2013). The notion of the importance of DQB1 matching may have risen in an era when high resolution typing of DRB1 was not yet universally in use. Considering the strong linkage disequilibrium between the two loci, DQB1 mismatches may actually have been an indirect indication of an allele level DRB1 mismatch.

Although practices in matching for DPB1 vary in different transplant programs, several retrospective studies have shown that some DPB1 mismatches are well tolerated (permissive) while others are not (non-permissive) (Zino *et al.*, 2004; Crocchiolo *et al.*, 2009; Fleischhauer *et al.*, 2012; Pidala *et al.*, 2014; Petersdorf *et al.*, 2015). Also, actual DPB1 match (12/12) has been shown to be beneficial in transplants performed for early leukemia (Shaw *et al.*, 2010), and it seems probable that also in transplants performed for non-malignant diseases, where graft versus leukemia effect is not required,12/12 match would be the optimal goal to keep the risk of GvHD as low as possible. The impact of non-permissive DPB1 mismatches has in most studies seemed somewhat smaller than mismatches in the A, B, C and DRB1 genes (Zino *et al.*, 2004; Fleischhauer *et al.*, 2012; Pidala *et al.*, 2014; Petersdorf *et al.*, 2015) but nevertheless significant. One smaller study with a more stringent division of DPB1 alleles resulted in a stronger impact, comparative to the high expression mismatches (Crocchiolo *et al.*, 2009).

Some studies have shown that individual allele level mismatches may be either better or worse tolerated than others (Kawase *et al.*, 2009; Fernandez-Viña *et al.*, 2014; Morishima *et al.*, 2016), but due to the HLA diversity, collecting data on individual mismatches is difficult and requires extremely large datasets. There have also been studies reporting that haplotype matching (E.W. Petersdorf *et al.*, 2007), frequent haplotypes (Jöris *et al.*, 2013) or ultra-high resolution matching between the patient and donor (Mayor *et al.*, 2019) are beneficial, showing that even now, optimal donor selection according to HLA only is not a straightforward process, and the optimal criteria may differ for different patients.

5.5 STEM CELL REGISTRIES

5.5.1 History and Development

The oldest of the present registries is Matchis in Holland, established in 1970 by the name Europdonor. In the beginning however, it provided unrelated bone marrow only occasionally, as its main function was to provide HLA matched platelets for HLA immunized patients (van Rood and Oudshoorn, 2008). The first registry that was established solely to provide unrelated hematological stem cells, Anthony Nolan, was founded in Great Britain in 1974 by Anthony Nolan's mother, Shirley Nolan. By the first half of 1980's 50 000 volunteers had been recruited and HLA typed by the Anthony Nolan registry (Blume, Forman and Appelbaum, 2004). Subsequently, France and the United States were among the next to start recruiting unrelated donors: France Greffe de Moelle Registry and the NMDP were both founded in 1986 (*https://bethematch.org/about-us/our-story/*, 2019; *https://www.dondemoelleosseuse.fr/france-greffe-moelle-registry*, 2019). Although the core of many registries early on were the previously HLA-A and -B typed blood donors, the families of patients who could not find a match among the existing donors were often active in recruiting new donors from their respective communities (Blume, Forman and Appelbaum, 2004; Margulies *et al.*, 2005).

As the outcomes of unrelated donor transplants were inferior to HLA identical sibling transplants, their number remained quite low in the beginning, as did the number of registered volunteer donors. In 1990, approximately 250 000 donors had been registered in the USA and Europe (Gajewski, Cecka and Champlin, 1990), and only 200 URD transplants were reported to have taken place so far (Gluckman, 1990). In the 1980's and early 1990's unrelated donors who were serologically HLA matched for HLA-A, -B and -DRB1 were considered sufficiently matched, and the size of unrelated donor registries was estimated accordingly: for patients with common HLA types, registries with 500-1000 donors were thought to suffice, whereas for patients with extremely rare HLA phenotypes the required donor pool was estimated to exceed a million donors (Gajewski, Cecka and Champlin, 1990).

The number of unrelated donor transplantations and registries increased rapidly during the 1990's. Germany, which at the moment harbors the largest unrelated donor registry in Europe, began active recruiting relatively late, in the beginning of 1990's (*www.ZKRD.de*, 2019); in Scandinavia, the Norwegian Bone Marrow Donor Registry was established in 1990 and both the Swedish Tobiasregistret and the Finnish Stem Cell Registry (former Finnish Bone Marrow Registry) in 1992. By the end of the 1990's, more than half a million new donors were recruited annually (Hurley *et al.*, 1999), so that in 2007 the number of donors worldwide exceeded 10 million, and in 2017 30 million. Also the use of unrelated donors has rapidly increased: in 2004, 7266 unrelated donors donated stem cells, of which 35 % cross country borders (Hurley *et al.*, 2007). Use of unrelated donors surpassed the use of sibling donors in 2006, and in 2012 more than 10 000 unrelated donor transplants crossed country borders (Gratwohl *et al.*, 2015).

5.5.2 Co-operation and International donor searches: BMDW and WMDA

Co-operation between registries and countries is a prerequisite for the international utilization of stem cell grafts. The core organizations for promoting this have been the Bone Marrow Donors Worldwide (BMDW) and the World Marrow Donor Association (WMDA).

BMDW was initiated by the Immunology Working Party of the EBMT in 1989, to collect the HLA phenotype data of the then existing unrelated donor registries. The initial four editions of the listings were distributed to registries on paper (Schipper *et al.*, 1996). In early 1990's, the database of worldwide registry donors and their HLA typing results was updated four times a year, and the data sent to the registries on either computer diskettes or on paper (Buskard and Stroncek, 1993). With the development of internet, the donor HLA data and searches were moved there with increasing data on donor characteristics like gender, age, cytomegalovirus (CMV) status and probability of HLA matching added over time (van Rood and Oudshoorn, 2008). Today, both registry donor data upload and registry donor searches are web-based.

In addition to facilitating searches for phenotypically HLA matched stem cell donors for individual patients, the large numbers of individuals from different ethnic groups contained in the database has also enabled HLA antigen and phenotype frequency analyses between different populations (Schipper *et al.*, 1996).

The WMDA was established in 1994 at an initiative of John Goldman (Jager, Brand and Claas, 2019). The idea was to develop guidelines to facilitate safe international exchange of hematopoietic stem cells. This is performed by several working groups, which cover quality assurance of donor registries, medical issues, ethics, and information technology. In 2003, an accreditation program for donor registries was established. The aim is to ensure uniform standards for international stem cell exchange.

In 2017 WMDA took over the activities of BMDW, and the worldwide donor searches are now performed at https://search.wmda.info (*https://search.wmda.info*, 2019). The database includes more than 35 million volunteer donors and almost 800 000 cord blood units, from 138 registries or cord blood banks from 53 different countries (*https://statistics.wmda.info*, 2019).

5.6 PLATELET TRANSFUSION REFRACTORINESS

5.6.1 Role of platelet transfusions in HSCT

The myeloablative conditioning regimens that precede HSCT destroy the patient's own bone marrow and therefore also intercept the production of megakaryocytes and platelets. Leukemia patients usually also undergo frequent cytopenias due to cytotoxic medications during their treatment before the stem cell transplant. After allogeneic stem cell transplant, the donor derived stem cells engraft and resume the production of blood cell lines. For platelets, the time of engraftment varies according to the graft source. The mean is 13 days for peripheral blood stem cells and 19 days for bone marrow (Holtick *et al.*, 2014).There is also variation according to the graft cell counts, composition and HLA matching (Delaney, Ratajczak and Laughlin, 2010; Patel *et al.*, 2018; Rimando *et al.*, 2018). Allogeneic HSCT patients are dependent on supportive care with allogeneic platelet transfusions until engraftment. Before platelet transfusions were implemented as standard care, a frequent complication of leukemia treatment and the single most common cause of mortality was hemorrhages (Freireich, 2000).

5.6.2 Assessment of platelet transfusion refractoriness

Today, repeated platelet transfusion support is a routine part of acute leukemia and post-HSCT treatment. However, up to 30 % of hematological patients are refractory to platelet transfusions (Hod and Schwartz, 2008), and these patients are in a significantly higher risk of serious bleeding events and death caused by bleeding

(Comont *et al.*, 2017). There are several different ways to assess platelet refractoriness, but the 1-h corrected count increment (CCI) is considered as an objective way to measure it (Hod and Schwartz, 2008); in clinical practice the easiest and most widely used estimate however is the absolute post transfusion count increment ACI, calculated simply as "Post-transfusion platelet count - Pre-transfusion platelet count"; this should normally be > 10 x 10^9/l in 24 h (Stanworth *et al.*, 2015). Usually more than one inferior transfusion increments are required before the patient is deemed transfusion refractory.

5.6.3 Platelet refractoriness due to non-immune causes

Febrile infections, bleeding, enlarged spleen, veno-occlusive disease, DIC (disseminated intravascular coagulopathy) and some medications cause excess platelet consumption, explaining most cases of clinical platelet refractoriness (Doughty *et al.*, 1994). However, increased consumption is more strongly reflected in the 24-h platelet increment, and immunological reasons are usually evident more quickly, by the 1-h increment (Daly *et al.*, 1980).

5.6.4 Immunological platelet transfusion refractoriness

Approximately 20 % of platelet refractoriness is caused mainly by immunological reasons (Doughty *et al.*, 1994; Legler *et al.*, 1997). The most common cause for these are pre-formed HLA antibodies, and a minority of patients also carry platelet specific (HPA) antibodies (Legler *et al.*, 1997). Presently HLA alloantibodies are most often caused by pregnancies and are also frequent among solid organ transplant recipients. When leukodepletion was not yet a universally applied measure, blood transfusions were another common trigger for alloimmunization. Leukodepletion does not totally remove the risk, but lowers it considerably (Slichter *et al*., 1997). All HLA antibodies do not manifest as platelet refractoriness: especially patients with lower levels of alloantibodies are reported not to develop a permanent clinical refractoriness (Hogge *et al.*, 1983).

5.6.5 Assessment of HLA antibody induced refractoriness

HLA antibodies have for decades been known to be responsible for hyperacute rejections of transplanted solid organs and also less acute rejections (Terasaki, Kreisler and Mickey, 1971; Soulillou, Peyrat and Guenel, 1978). Because of their critical role in solid organ transplantation, different pre-transplant testing methods for these antibodies have been developed (Bray and Gebel, 2009). The same methods can be utilized for testing the HLA antibody status of platelet transfusion refractory patients as well (Peña and Saidman, 2015). Today, the most widely used method, Luminex, is based on a micro bead array. This method utilizes differently colored microbeads, which are covered with known HLA molecules. The pre-formed antibodies of the patient bind to their specific target molecules, leaving the rest of the beads intact. The binding of antibodies on the specific beads is then detected using secondary antibodies and fluorescence. The binding of the antibodies is semi-quantitatively measured and given as mean fluorescence intensity (MFI) (Lachmann *et al.*, 2013). Luminex method is both sensitive, more specific and less labor intensive than the earlier complement dependent cytotoxicity testing (CDC) methods (Wehmeier, Hönger and Schaub, 2019). When the antibody status of the patient has been analyzed, the estimated width of the patient's immunization can be reported as the percentage of panel reactive antibodies (PRA %). PRA % depicts the likelihood of any random donor carrying HLA antigen(s) against which the patient carries an HLA antibody.

5.6.6 Management of immunological platelet transfusion refractoriness

To overcome immune mediated refractoriness, platelet refractory patients are usually transfused with specifically selected platelets, and the selection is based on one of several published methods. It is still unclear which of the methods is superior, and many blood services use a combination of selection methods (Pavenski, Freedman and Semple, 2012). The most common methods are 1) cross-matching of platelets; 2) using blood donors who carry HLA antigens that are identical with the patient's HLA

antigens or resemble them as closely as possible; 3) avoidance of HLA antigens against which the patient carries HLA antibodies; 4) combination of the previous two methods. (Stanworth *et al.*, 2015). The majority of blood services utilize the patient's antibody profile (Jouni Lauronen, personal information), but the recommended MFI cut-off values for the patient's HLA antibodies are not well established. The similarity between the donor's and patient's HLA antigens on the other hand can be assessed using either the older method of cross reactive groups of HLA antigens, or an excel-based algorithm, HLAMatchmaker program, (Duquesnoy, 2006, 2008), which measures the structural similarity between the patient's and donor's HLA antigens as a number of eplet mismatches. Eplets are described as patches of polymorphic residues, situated close to each other in the HLA molecule, and in antibody accessible positions.

6 AIMS OF THE STUDY

The goal of this thesis was to optimize some very practical HLA typing and donor selection related aspects of the work at HLA laboratories and unrelated stem cell donor registries, so that they would be able to give better and more reliable service to the hematological transplant centers, and via them, the life-threateningly ill HSCT patients.

The specific aims of the individual parts of the study were:

1) To estimate and minimize the risk of erroneous HLA typing results for leukemia patients who are HLA typed while carrying a high leukemic burden, and whose malignant cells carry LOH of the HLA region.

2) To describe the specific features of the Finnish HLA landscape and assess the utility of maintaining a Finnish stem cell registry, especially for Finnish hematological patients.

3) To describe Finnish 6-locus HLA haplotypes and assess the feasibility of predicting DPB1 match or DPB1 mismatch permissiveness of registry donors for individual patients during registry donor search.

4) To assess donor specific HLA antibody cut-off levels for safe and effective platelet transfusions of HLA alloimmunised hematological patients, a considerable proportion of whom are considered for HSCT and require repeated platelet transfusion support.

7 MATERIALS AND METHODS

7.1 STUDY SUBJECTS

I The discovery set consisted of five leukemia patients with an HLA allele loss of heterozygosity phenomenon which was identified during a routine HLA typing process. In addition, a retrospective HLA phenotype analysis was performed for 2893 hematological patients and a control group of 2895 healthy siblings and solid organ transplant patients, to assess a potential difference between HLA homozygosity frequencies between the groups. 65 patients, previously genotyped as homozygous for at least two adjacent HLA loci and considered potentially suspect of undetected LOH, were chosen for HLA retyping by SSP, to confirm the initial typing result.

II Finnish high resolution 5-locus HLA haplotype frequencies were assessed from the Finnish Stem Cell registry database, and 9774 registry members were included in the analysis. HLA haplotype frequencies for Sweden, Russia and Germany were estimated from the Tobias Registret, HPC Registry Russia Samara and ZKRD, including 21797, 7855 and 2989989 individuals, respectively. To assess the coverage of Finnish, Swedish and German registries for Finnish patients, virtual match predictions in each registry were performed, using HLA phenotypes of 1492 Finnish patients. For the actual HSCT donor search and matching analysis, 647 consecutive Finnish donor searches, ending up in 416 stem cell transplantations, were retrospectively analyzed.

III Finnish high resolution 6-locus HLA haplotype frequencies were assessed from a group of 43 365 current or former members of the Finnish Stem Cell Registry; registry members residing in Estonia were excluded. 15 288 of Finnish registry members had been typed for all six genes. The DPB1 matching data of unrelated donor transplants was assessed from 769 10/10 matched URD transplants that had been performed between 2003-2016, separately for patients who had received their transplant from a domestic donor and those with a foreign donor.

IV The 270 platelet transfusion responses of 40 adult hematological patients, transfused with HLA selected platelets between 2008 and 2011, were included in the study. To be included, the patient had to be treated in one of the five Finnish university hospitals, and both the patients' immunization status and transfusion responses had to be recorded. In addition to these 40 hematological patients, the frequency of HLA alloimmunization in a separate group of 108 consecutive platelet refractory patients was retrospectively assessed for the study. The HLA class I antibody status of these patients had been analyzed in the FRCBS HLA laboratory at a later period, between 2013 and 2016.

7.2 HLA TYPING (I-IV)

Most of blood donors (study IV) and approximately 40 % of registry donors in study II were low resolution typed by serological assays (Biotest Rockaway, NJ, USA).

The low resolution typing of most patients and some registry donors (studies I-IV) was performed by rSSO-Luminex technology (Labtype, One Lambda inc. Canoga Park, CA). rSSO-Luminex technology was used also for some patient's and registry donors' high resolution HLA-DQ and HLA-DP-typing. The Luminex results were analyzed with Fusion software (One Lambda, Inc. Canoga Park, CA).

Most of the patients' and HSCT donors' high resolution typing (I-III) was performed with sequence-based typing (Atria Genetics, Hayward, CA) using the ABI 3130xl genetic analyzer (Applied Biosystems, Thermo Fisher Scientific, MA) and analyzed with the Assign 3.5+ software (Conexio Genomics Pty Ltd, Fremantle, Australia) according to the providers instructions.

For confirmatory HLA typing in suspected LOH cases (study I), the polymerase chain reaction (PCR) SSP method was used (Micro SSP™ Generic HLA Class I/II DNA Typing Trays, One Lambda Inc. Canoga Park, CA; Olerup SSP® genotyping, Olerup SSP AB, Stockholm, Sweden). The PCR-SSP was run on 2.0% agarose gel and analyzed with the Fusion software.

The majority of DPB1 typed registry donors (study III) were typed by SBT using Sanger sequencing and next generation sequencing techniques by a commercial service provider (Histogenetics inc. 300 Executive Blvd, Ossining, NY 10562 USA).

7.3 HLA ANTIBODY TESTING (IV)

HLA Luminex-based methods were used for HLA alloantibody detection (Luminex Corporation, USA). The serum samples of patients were first screened either by LABScreen Panel reactive antibody (PRA) assay or LabScreen Mixed screening assay (One Lambda). After positive screening for HLA class I, the antibody specificities were assessed by the LABScreen IgG single antigen assays (OneLambda) measured on a LABScan100 flow cytometer platform. The MFI values of individual antibodies were determined with HLA Fusion software according to the manufacturer's default settings. PRA % was calculated from the single antigen antibody analysis, using a Finnish population specific panel.

7.4 CLINICAL DATA (I, IV)

I: The clinical background data of five LOH patients was individually provided by the treating physician of each patient.

IV: Clinical data for the patients and transfusions were collected from the Finnish university hospitals retrospectively with a questionnaire. The pre- and post-transfusion platelet counts and the respective levels of the patient's platelet donor specific HLA antibody levels were individually assessed for altogether 270 HLA selected platelet transfusions of the 40 patients.

7.5 DONOR SEARCH AND STEM CELL TRANSPLANTATION DATA (II, III)

In study II, 649 consecutive registry donor searches performed at a request of Finnish transplant centers during 2010 - 2014 were analyzed. During this period the searches were automatically saved at the Bone Marrow Donors Worldwide search database and could thus be retrospectively retrieved.

Data on patient-donor pairs for all unrelated donor HSCTs facilitated by the Finnish Stem Cell Registry, and the HLA data of the patients and their respective donors and donor candidates are recorded in the Velho laboratory database of the histocompatibility laboratory of the Finnish Red Cross Blood Service, and were retrieved accordingly (studies II and III).

The HLA haplotype combinations of all patients were assessed and an analysis made based on existing family HLA typing data (II and III) or, in the absence of HLA typed relatives, using estimated Finnish HLA haplotype frequencies (II). The patients were divided into two groups according to the result of the analysis: either likely or not likely to carry at least one HLA haplotype specifically enriched in the Finnish population, and rare elsewhere. For Finnish registry donors, a similar division was done, based solely on the estimated HLA haplotype frequencies (II).

7.6 BIOINFORMATICS AND STATISTICAL ANALYSIS

I-III

Haplotype frequencies (HF) for 3-locus HLA-A, -B, -DR low (LR) and 5-locus HLA-A, -B, -C, -DRB1, -DQB1 high resolution (HR) were estimated using ZKRD's haplotype frequency estimation (HFE) algorithm (Eberhard *et al.*, 2013), that is based on the Expectation-Maximization (EM) method.

The genetic distance between two donor populations (DF) was assessed using Prevosti's metric

$$D_1(f,g) = \frac{1}{2}\sum_i |f_i - g_i|$$

and the homogeneity of the populations using the Simpson index $\lambda = \sum_i f_i^2$.

Two-tailed Fisher's exact test (*https://www.graphpad.com/quickcalcs/catMenu/*) was used in the statistical comparisons between the frequency of homozygosity between patients and controls in the LOH study, DPB1 allele matching of domestic versus

foreign donors in the extended haplotype study, FER (Finnish enriched rare) haplotype positive and negative patients, and FER positivity between exported stem cell transplants and HSCT patients in the HLA isolate study.

Fisher's exact test was also used for comparing the haplotype-specific DPB1 allele frequency to the frequency of the same allele in the study population (n=30576). The p-values were adjusted for the total number of conducted tests by the Bonferroni method. The data were analyzed and plotted using R v3.4.4(The R Core Team, 2018) with libraries *ggplot2* v3.1.0(Wickham, 2016), *data.table* v1.11.8(Dowle and Srinivasan, 2018), and *gplots* v3.0.1(Warnes *et al.*, 2016). The haplotype-against-DP hierarchical clustering was generated using the *heatmap.2* function with default settings.

IV

The Chi-Square test and Fisher's exact two-tailed test were used for examining the relationship between two categorical variables. The absolute platelet count increment at 18-24 hours was modeled using a multivariable mixed effect model, where a patient was considered a random effect. The multivariable model was fitted in R 3.1.1 (R Core Team, 2014) using the add-on package nlme (Pinheiro *et al.*, 2014). In addition, the probability of a good absolute platelet count increment (ACI>15 at 18-24 h post-transfusion) was modelled using a logistic regression using the R software. Receiver operating characteristic curve (ROC) was used for determining an optimal threshold for maximizing the accuracy of the prediction based on the predictions from the fitted logistic regression model.

7.7 ETHICS

The Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District approved the study protocol 42/13/03/00/11. The Medical Ethics Committee of the Helsinki and Uusimaa Hospital District approved the study protocol 305/13/03/01/2011.

8 RESULTS

8.1. LOSS OF HETEROZYGOSITY OF THE HLA REGION (I)

8.1.1 Differences between HLA typing methods in detecting LOH of the HLA complex

At the time of the study, we had encountered five patients with a loss of heterozygosity at HLA. All patients had been diagnosed with a hematological malignancy: three with AML, one with CLL and one with a lymphoma that had transformed into leukemia. The first identified case of LOH in our laboratory was observed in an AML patient whose confirmatory HLA typing sample was heterozygous for HLA-A and HLA-C locus, in contrast to homozygosity of all loci in the initial sample two months previously. As the heterozygous C locus was inconsistent with a homozygous B locus, a third sample was requested and drawn two weeks later, and only then was the B locus also shown to be heterozygous. At the time of the HLA typing of the four subsequent LOH patients, HLA LOH as a phenomenon was already better recognized and the assignment of the final heterozygous results less dramatic, but still far from straightforward. The proportion of leukemic cells in their peripheral blood at the time of HLA typing varied from 55 % for the CLL patient to 93 % for one of the AML patients. None of the patients had carried chromosome 6 deletions detectable by routine karyotyping, suggesting copy neutral loss of heterozygosity.

To achieve reliable HLA assignment, all patients had been typed with a combination of different methods. During the typing processes of these patients we repeatedly observed that the SSP method could confirm the HLA heterozygosity of the patient, whereas it could be suspected (rSSO background reactivity) or missed altogether

(Sanger sequencing) by the primary methods. Figure 3 depicts examples of an rSSO and a sequencing analysis of a LOH patient's HLA-B.

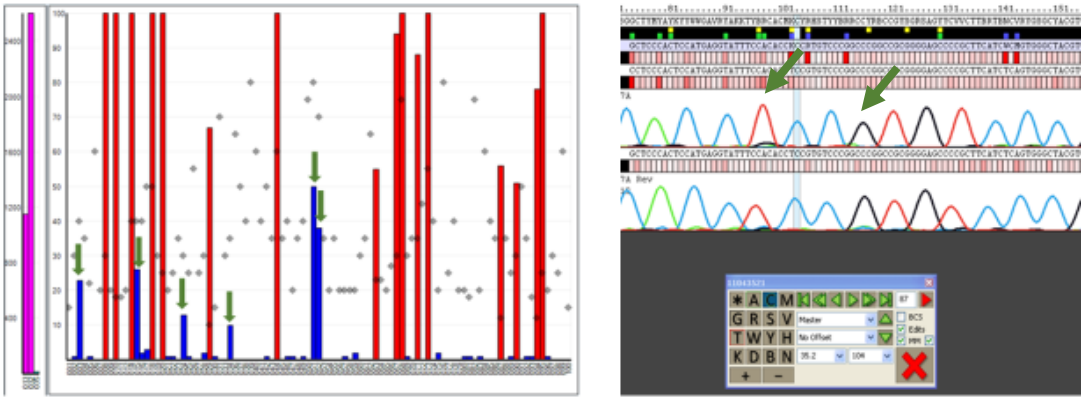

Figure 3. *HLA-B typing result of a LOH-patient. Left: Luminex SSO analysis. The red columns have been assigned "positive" by the Fusion software, and belong to the remaining allele. The blue columns are assigned "negative" by the software, with the six higher blue columns, here marked with green arrows, all belonging to the "lost" allele, while the very low blue columns are unspecific noise and do not belong to either allele. Right: DNA sequence analysis by Sanger sequencing of HLA-B of the same patient. The two heterozygous peaks (green arrows) are almost impossible to discern.*

In the study, the observed sensitivities of the three available HLA typing methods were confirmed by creating an artificial LOH: two samples homozygous for the HLA-A gene were mixed together in varying proportions. The results show that the SSP method is the most sensitive in detecting heterozygosity: with it, a mix containing only 2.5% or the minor sample gave a sufficient signal for detecting both alleles. The threshold with rSSO was 5%–17% when the manufacturer's default

settings were used, and as high as 17%–33% with Sanger sequencing (Table 1). However, the threshold of rSSO can be lowered by taking into account the background reactivity, apparent in Figure 3 (left).

Proportion of the minor sample	**Sequencing (High resolution)**	**Luminex SSO (Intermediate resolution)**	**SSP (Low resolution)**
33%	Detects both alleles	Detects both alleles	Detects both alleles
17%	Detects only one allele	Detects both alleles	Detects both alleles
5%	-"-	Detects only one allele	Detects both alleles
2,5%	-"-	-"-	Detects both alleles
1%	-"-	-"-	Detects only one allele
0,5%	-"-	-"-	-"-

Table 1. *The differences between typing methods in detecting heterozygosity with an HLA-A*02-homozygous sample diluted with an HLA-A*24-homozygous sample.*

8.1.2 Prevalence of LOH with implications for HLA typing

Five patients with LOH among the 950 hematological patients that had been HLA typed in our laboratory between 2011 and 2013 indicate a frequency of 0.4% for LOH in our material. As most previous publications only reported individual LOH cases, the true frequency of LOH phenomenon strong enough to endanger correct HLA typing, was unknown. There were no previously recognized LOH cases in FRCBS HLA laboratory before the five that we reported, and it seemed quite possible that

some mistyped LOH cases might previously have gone unnoticed. To check this, 2893 hematological patients, HLA typed between 2003 and 2012, and a control group of 2895 healthy siblings and solid organ transplantation patients were first screened for HLA homozygosity. The frequency of HLA homozygosity between hematological patients and the control group was compared, and no statistically significant difference was observed (Table 2).

	All	HLA-A-B or HLA-B-DRB1 homozygous		HLA-A-B-DRB1 homozygous	
	Number	number	%	number	%
Hematological patients	2893	108	3,7	79	2,7
Control group	2895	118	4,1 (p=0,54)	75	2,6 (p=0,74)

Table 2. *Individuals homozygous for two or three adjacent HLA gene loci, and the proportions of HLA homozygotes among hematological patients and a control group. The control group consists of their siblings (n= 1365), solid organ transplantation patients (n= 1468) and related donors of kidney transplantation patients (n=62).*

Additionally, 65 of the 2893 hematological patients were retrospectively chosen for confirmatory typing by low resolution SSP, to exclude previously undetected LOH. All 65 were homozygous for a single rare allele or two to three adjacent HLA loci,

had no family data confirming the homozygosity, and had either been HLA typed only once or the two samples had been drawn less than two months apart. Re-typing with SSP revealed no additional LOH cases among this group. The results indicate that in patients who are HLA typed for hematological reasons, the frequency of HLA LOH that is grave enough to cause HLA mistyping, is 0.4 % at most. Since 2012, we have however encountered altogether 13 such cases among the 2620 HLA genotypings performed for hematological patients, resulting in a frequency of 0.49 %, and averaging 1-2 cases annually (unpublished observation).

The probability of LOH inflicting HLA mistyping depends obviously not only on the frequency of LOH itself, but also on the proportion of affected cells in the HLA typing sample and the typing methods that have been used. The fact that no mistyped HLA analyses were detected before 2012 may thus be partly due to the characteristics of older typing methods that detect the "lost" HLA easier; it is also conceivable that the apparent increase of HLA LOH is due to different national protocols for HLA sampling. It is now included in the set of protocol blood tests that are drawn at the very outset of leukemia diagnosis, the exact point of time when the faulty typing result is most likely.

As the proportion of aberrant cells in the sample must be substantial to inflict problems with HLA typing, it is likely that even today a number of HLA-associated LOH-phenomena go undetected. When there is only a minor imbalance between the HLA alleles in the sample, an upfront heterozygous HLA assignment even with the manufacturer's default settings is likely. On the other hand, it is also possible that the proportion of aberrant cells in peripheral blood is so high that none of the common typing methods is able to detect the missing alleles. In such cases re-sampling HLA homozygous patients by either a buccal swab or at remission is the only way to confirm the heterozygous phenotype.

8.2 CHARACTERISTICS OF THE FINNISH HLA ISOLATE (II-III)

8.2.1 Comparisons to Sweden, Russia Samara and Germany (II)

The HLA haplotype frequencies of Finland, Sweden, Germany and Russia Samara were estimated using registry donor HLA data and ZKRD's EM algorithm. Although most of the individuals in the registries have been HLA typed only once, it is improbable that potential typing errors should have a significant effect on the estimated haplotype frequencies: the reported proportion of discrepant results among the WMDA member registries has been well under 1 % and in Finland < 0.5 % (Anne Arvola, FSCR, personal communication). A recent publication from the DKMS showed that with modern NGS assays, the frequency of typing discrepancies is actually as low as, 0.012-0.019 % (Baier *et al.*, 2019).

The resulting HLA haplotype frequencies of the four registries were compared with each other, first by the Simpson index, depicting the homogeneity of the population. Finnish HLA was by far the most homogenous of the four studied populations, by both low and high resolution (Figure 4). Russia Samara was the most heterogeneous, although Russian high resolution frequencies could not be estimated due to the low number of high resolution typed donors in the registry.

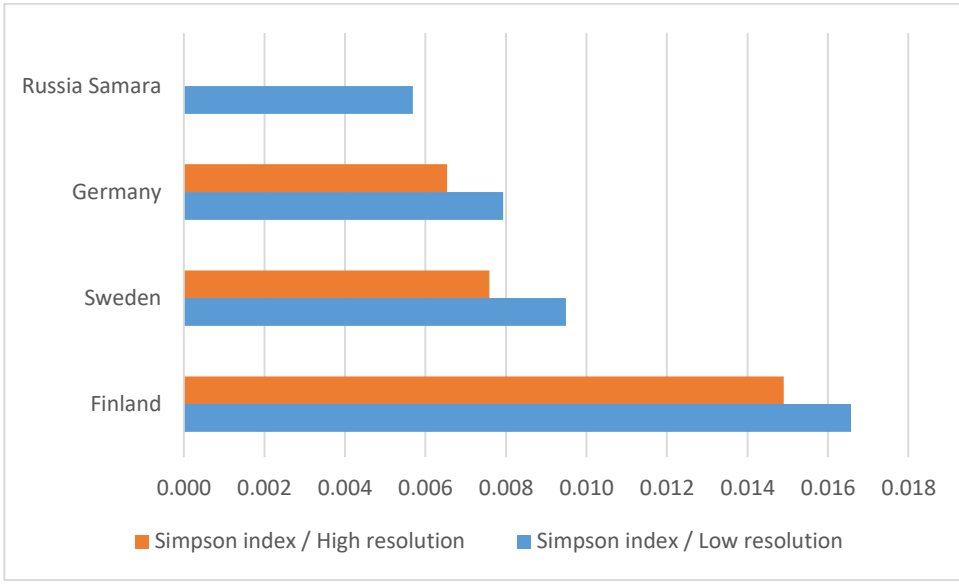

Figure 4. *The HLA homogeneity of the four studied populations, depicted by the Simpson index. The higher the index, the more homogenous the population; the index values can vary from zero to one.*

The genetic distances between the populations were assessed using both low and high resolution haplotype frequencies. The distance was shortest between Sweden and Germany, and longest between Finland and Russia (Figures 5a and 5b); the population nearest Finland was Sweden, which was not surprising, considering the long history of Finland as the eastern part of Sweden.

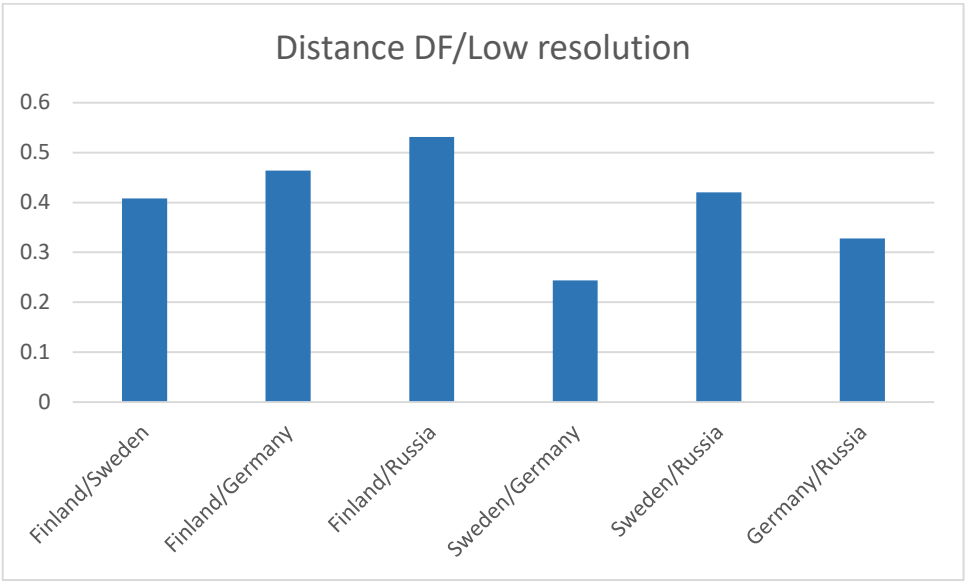

Figure 5a. *The genetic distances (low resolution HLA haplotype frequencies) between the four studied populations.*

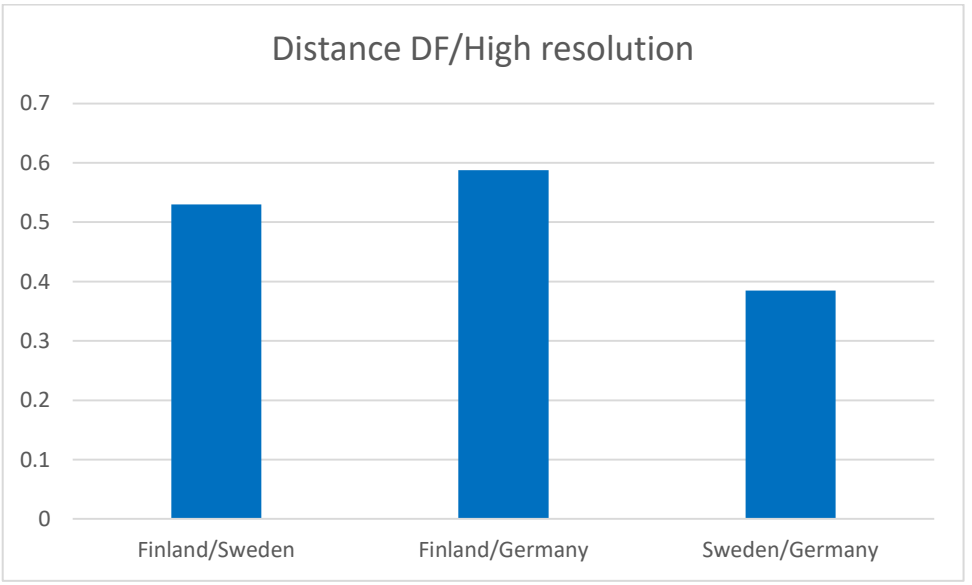

Figure 5b. *The genetic distances (high resolution haplotype frequencies) between the four studied populations.*

8.2.2 FER haplotypes (II and III)

Finnish haplotype frequencies were estimated from the Finnish registry donor data retrieved from the Bone Marrow Donors Worldwide database at the turn of the year 2014-2015 (study II), and Finnish registry donor data in the Prometheus database in 2018 (study III). The results of both studies show that the Finnish HLA landscape has a dualistic character. HLA allele variation is limited, and individual HLA alleles and the majority of the 5-locus haplotypes are commonly found in all Europeans (Table 3); on the other hand, approximately one third of the Finnish 5-locus HLA haplotypes are much more frequent in Finland than in other populations with known HLA-haplotype frequencies. The frequencies of the hundred most frequent Finnish HLA haplotypes and their respective frequencies in Germany and among the NMDP Caucasians are depicted in Figure 6 and Figure 7. For comparison, the hundred most frequent Swedish HLA haplotypes with their respective frequencies in Germany (Figure 8), show that on average, the Swedish frequencies are much closer to the German frequencies, with only some individual exceptions. Among the hundred most frequent Finnish 5-locus haplotypes there were, in both studies, 34 haplotypes with a frequency lower than 0.0003 in both the German registry donor pool and NMDP Caucasians, and with an at least 8-fold higher frequency in Finland. We named such haplotypes Finnish enriched rare or “FER”-haplotypes. Of the FER haplotypes, 30 were identical for both studies, but four in the earlier study were replaced by different ones in the later study, showing that Finnish enriched haplotypes extend beyond the hundred most frequent (Table 3).

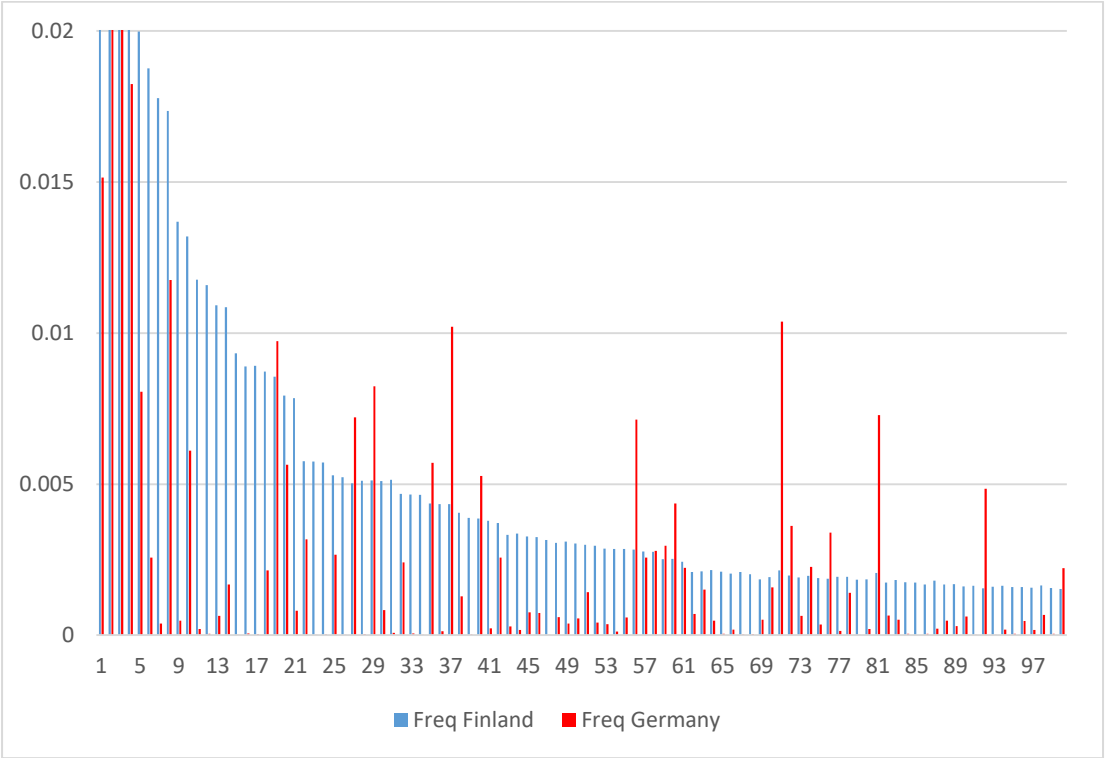

Figure 6. *The frequencies of the hundred most frequent Finnish 5-locus HLA haplotypes compared to their frequencies in Germany. To better depict the differences between the haplotypes outside the four most frequent, x-axis has been cut off at 0.02.*

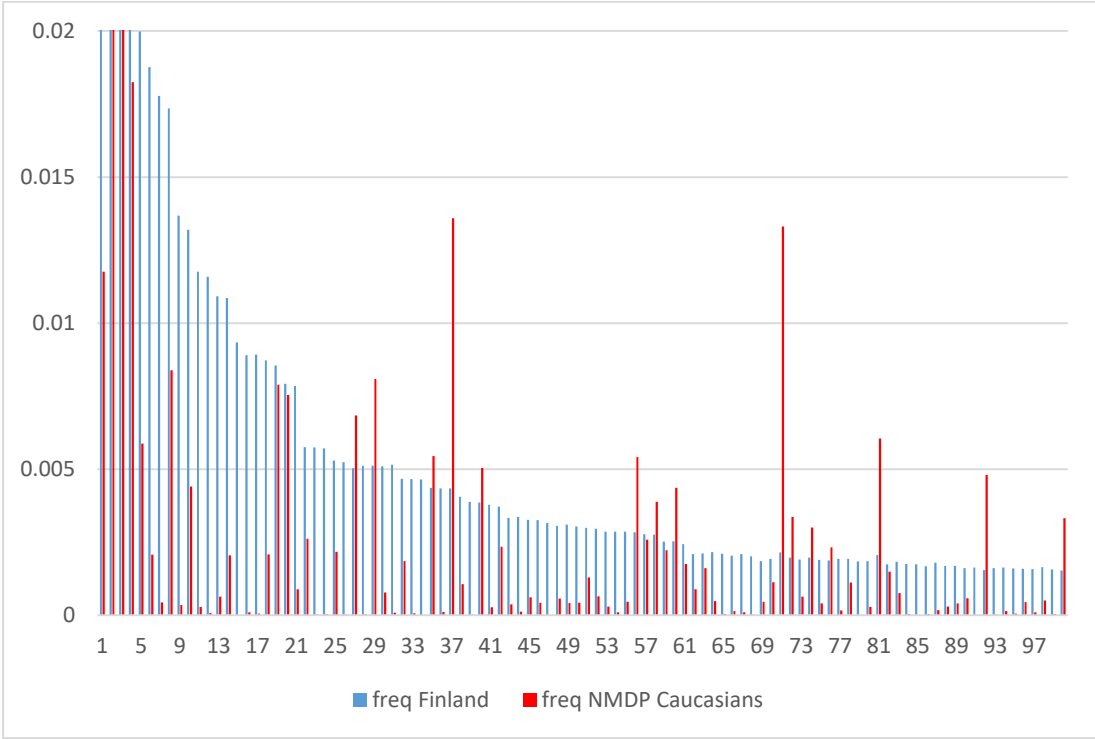

Figure 7. *The frequencies of the hundred most frequent Finnish 5-locus HLA haplotypes compared to their frequencies among NMDP Caucasians. To better depict the differences between the haplotypes outside the four most frequent, x-axis has been cut off at 0.02.*

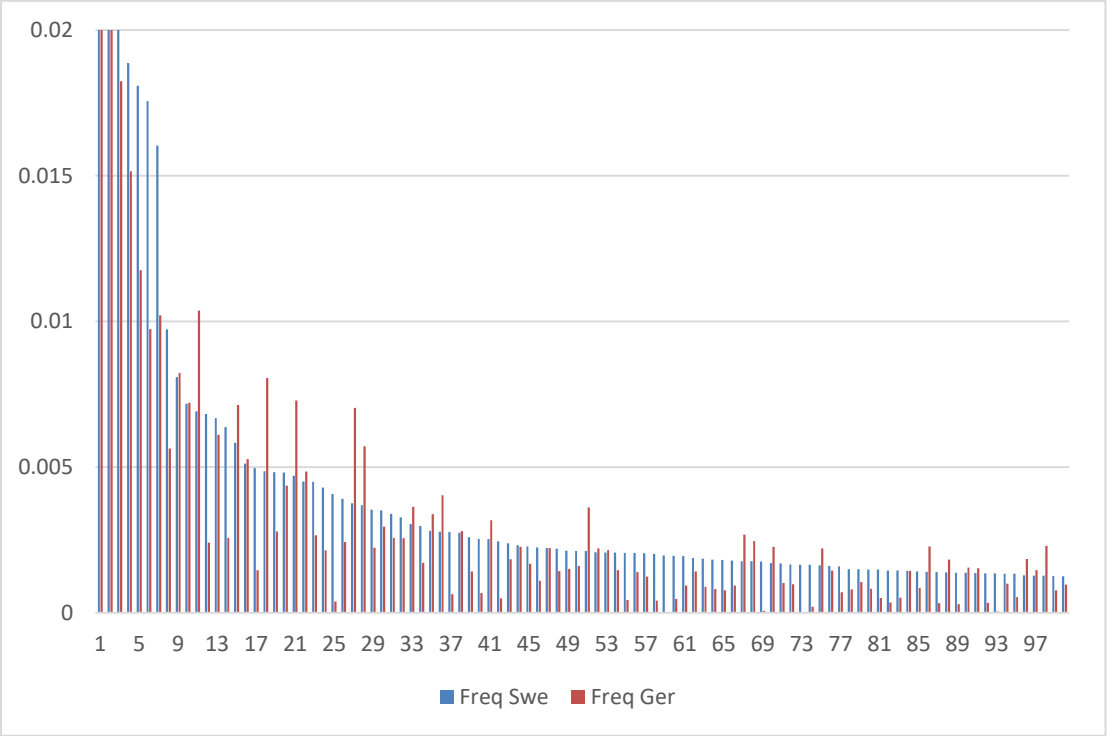

Figure 8. *The frequencies of the hundred most frequent Swedish 5-locus HLA haplotypes compared to their frequencies in Germany. To better depict the differences between the haplotypes outside the three most frequent, x-axis has been cut off at 0.02.*

It is of note that although the majority of Swedish HLA haplotype frequencies resemble the German frequencies, among the hundred most frequent Swedish HLA haplotypes there were four haplotypes fulfilling identical "SER" (Swedish enriched rare) criteria. Two of these four are shared by Finland and Sweden, being FER haplotypes as well, but two are specific for Sweden. One of the Swedish specific haplotypes (A*02:06, B*35:03, C*04:01, DRB1*13:02, DQB1*06:04) didn't have a reported frequency in Germany at all, thus being less frequent in Germany than any of the 34 FER haplotypes.

Rank 2018	Rank 2014	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1	Freq 2018	Freq 2014
1	1	03:01	35:01	04:01	01:01	05:01	0.083115	0.081422
2	2	01:01	08:01	07:01	03:01	02:01	0.0492	0.053315
3	3	03:01	07:02	07:02	15:01	06:02	0.033665	0.034424
4	4	02:01	07:02	07:02	15:01	06:02	0.022705	0.02222
5	6	02:01	13:02	06:02	07:01	02:02	0.01993	0.018392
6	5	03:01	07:02	07:02	13:01	06:03	0.018766	0.019849
7	8	02:01	27:05	02:02	08:01	04:02	0.017792	0.017152
8	7	02:01	15:01	03:04	04:01	03:02	0.01726	0.017635
9	9	02:01	15:01	04:01	08:01	04:02	0.013618	0.013955
10	10	02:01	15:01	03:03	13:01	06:03	0.013159	0.013799
11	12	**02:01**	**51:01**	**15:02**	**09:01**	**03:03**	0.011768	0.011514
12	13	**31:01**	**18:01**	**07:01**	**15:01**	**06:02**	0.01159	0.011059
13	11	24:02	40:01	03:04	13:02	06:04	0.010938	0.011526
14	15	02:01	27:05	01:02	01:01	05:01	0.010848	0.010244
15	17	**03:01**	**07:02**	**03:04**	**01:01**	**05:01**	0.009375	0.008914
16	16	**03:01**	**18:01**	**07:01**	**04:04**	**03:02**	0.008912	0.009103
17	19	**03:01**	**15:01**	**03:03**	**08:01**	**04:02**	0.008886	0.008115
18	21	02:01	44:02	05:01	12:01	03:01	0.008674	0.00765
19	20	02:01	40:01	03:04	13:02	06:04	0.008525	0.007881
20	18	02:01	08:01	07:01	03:01	02:01	0.007999	0.008408
21	14	68:01	08:01	07:01	03:01	02:01	0.007855	0.010276
22	28	02:01	35:01	04:01	01:01	05:01	0.005803	0.005713
23	29	**02:01**	**27:05**	**01:02**	**04:08**	**03:01**	0.005795	0.005514
24	23	**24:02**	**39:01**	**07:02**	**04:04**	**03:02**	0.005756	0.005972
25	27	02:01	40:01	03:04	08:01	04:02	0.005236	0.005726
26	30	**24:02**	**39:01**	**07:02**	**08:01**	**04:02**	0.005213	0.005344
27	33	24:02	07:02	07:02	15:01	06:02	0.005152	0.004914
28	22	**32:01**	**39:01**	**07:02**	**08:01**	**04:02**	0.005136	0.00608
29	25	01:01	57:01	06:02	07:01	03:03	0.005106	0.005921
30	32	24:02	35:01	04:01	01:01	05:01	0.005097	0.004919
31	24	**02:01**	**56:01**	**01:02**	**04:01**	**03:02**	0.005081	0.005936
32	31	03:01	15:01	03:04	04:01	03:02	0.00469	0.004997
33	34	**24:02**	**40:01**	**03:04**	**09:01**	**03:03**	0.004683	0.004563
34	36	**31:01**	**51:01**	**01:02**	**13:01**	**03:03**	0.004643	0.004217
35	40	11:01	35:01	04:01	01:01	05:01	0.004449	0.003926
36	37	**03:01**	**47:01**	**06:02**	**15:01**	**06:02**	0.00437	0.004165
37	38	02:01	44:02	05:01	04:01	03:01	0.004318	0.004042
38	41	02:01	40:02	02:02	11:01	03:01	0.004002	0.003919
39	26	**68:01**	**35:01**	**03:03**	**08:01**	**04:02**	0.003923	0.00591
40	42	25:01	18:01	12:03	15:01	06:02	0.003844	0.003576
41	48	**32:01**	**44:02**	**05:01**	**04:01**	**03:02**	0.003775	0.003083
42	35	02:01	15:01	03:03	04:01	03:02	0.003755	0.004426
43	46	24:02	15:01	03:03	01:01	05:01	0.003351	0.003199

44	47	**02:01**	**56:01**	**01:02**	**15:01**	**06:02**	0.00334	0.003131
45	45	25:01	18:01	12:03	01:01	05:01	0.003332	0.003246
46	53	03:01	44:27	07:04	16:01	05:02	0.003285	0.002696
47	44	**03:01**	**27:05**	**01:02**	**04:08**	**03:01**	0.003178	0.003302
48	73	24:02	44:02	05:01	04:01	03:01	0.003067	0.001938
49	51	24:02	07:02	07:02	13:01	06:03	0.003027	0.002904
50	60	32:01	15:01	03:03	11:01	03:01	0.003001	0.0024
51	56	02:01	40:01	03:04	01:01	05:01	0.002985	0.002517
52	58	03:01	27:05	01:02	01:01	05:01	0.002967	0.002437
53	49	03:01	35:01	04:01	04:01	03:02	0.002936	0.003072
54	63	**02:01**	**15:01**	**04:01**	**15:01**	**06:02**	0.002916	0.002314
55	39	68:01	51:01	14:02	13:01	06:03	0.002877	0.003974
56	55	02:01	57:01	06:02	07:01	03:03	0.002869	0.002623
57	43	03:01	08:01	07:01	03:01	02:01	0.002781	0.003332
58	50	02:01	44:02	05:01	15:01	06:02	0.002634	0.002984
59	52	24:02	15:01	03:03	13:01	06:03	0.002521	0.002705
60	54	02:01	44:02	05:01	13:01	06:03	0.002504	0.002677
61	71	02:01	27:05	02:02	01:01	05:01	0.002472	0.001956
62	83	02:01	35:01	04:01	14:01	05:03	0.00216	0.001704
63	64	01:01	37:01	06:02	10:01	05:01	0.002125	0.002291
64	90	02:01	40:01	03:04	09:01	03:03	0.002117	0.001623
65	68	**02:01**	**39:01**	**12:03**	**09:01**	**03:03**	0.002113	0.002109
66	59	**02:01**	**15:01**	**03:04**	**08:01**	**04:02**	0.002107	0.002402
67	70	**31:01**	**27:05**	**01:02**	**04:04**	**03:02**	0.002079	0.002054
68	86	**02:01**	**13:02**	**06:02**	**09:01**	**03:03**	0.002015	0.001657
69	115	02:01	51:01	15:02	13:01	06:03	0.00196	0.001386
70	66	03:01	15:01	03:03	13:01	06:03	0.001945	0.002204
71	69	29:02	44:03	16:01	07:01	02:02	0.001933	0.00208
72	95	03:01	07:02	07:02	01:01	05:01	0.001928	0.00157
73	82	01:01	15:01	03:03	13:01	06:03	0.001928	0.001704
74	76	11:01	07:02	07:02	15:01	06:02	0.001908	0.001847
75	80	68:01	40:01	03:04	04:04	03:02	0.001884	0.001737
76	65	68:01	44:02	07:04	11:01	03:01	0.001881	0.002211
77	57	**03:01**	**15:01**	**03:03**	**01:01**	**05:01**	0.001873	0.002496
78	78	02:01	07:02	07:02	13:01	06:03	0.001855	0.001799
79	79	**29:01**	**41:01**	**17:01**	**07:01**	**03:03**	0.001826	0.001746
80	85	**11:01**	**44:02**	**05:01**	**12:01**	**03:01**	0.001815	0.001664
81	105	23:01	44:03	04:01	07:01	02:02	0.001783	0.001465
82	84	02:01	44:02	05:01	07:01	02:02	0.001777	0.001698
83	62	02:01	27:05	02:02	04:04	03:02	0.001773	0.002322
84	118	***11:01***	***44:02***	***05:01***	***08:01***	***04:02***	0.00176	0.00133
85	154	***32:01***	***40:02***	***03:04***	***14:02***	***03:01***	0.001733	0.001016
86	101	***03:01***	***15:01***	***03:03***	***13:02***	***06:04***	0.001703	0.001478
87	74	**03:01**	**35:01**	**04:01**	**04:01**	**03:01**	0.0017	0.001911
88	103	31:01	39:01	12:03	12:01	03:01	0.001687	0.001475

89	88	02:01	27:05	01:02	08:01	04:02	0.001648	0.001635
90	96	24:02	38:01	12:03	13:01	06:03	0.001643	0.001557
91	116	***26:01***	***40:02***	***03:04***	***08:02***	***04:02***	0.00164	0.001371
92	110	01:01	07:02	07:02	15:01	06:02	0.001628	0.001422
93	89	**68:01**	**51:01**	**14:02**	**03:01**	**02:01**	0.001614	0.001632
94	81	**03:01**	**51:01**	**14:02**	**13:01**	**06:03**	0.001612	0.001707
95	77	**02:01**	**18:01**	**12:03**	**01:01**	**05:01**	0.001611	0.001829
96	87	24:02	15:01	03:03	04:01	03:02	0.001582	0.001644
97	91	**24:02**	**13:02**	**06:02**	**10:01**	**05:01**	0.001579	0.001617
98	93	02:01	15:01	03:04	13:01	06:03	0.001571	0.001592
99	61	**68:01**	**38:01**	**12:03**	**15:01**	**06:02**	0.001565	0.002337
100	139	30:02	18:01	05:01	03:01	02:01	0.001531	0.001125
101	98	**26:01**	**15:01**	**04:01**	**01:01**	**05:01**	0.00153	0.00154
102	100	**03:01**	**40:02**	**03:04**	**14:02**	**03:01**	0.00151	0.001478
103	148	01:01	35:01	04:01	01:01	05:01	0.001476	0.001054
104	109	32:01	35:01	04:01	04:01	03:01	0.001475	0.001432
105	94	02:01	18:01	07:01	15:01	06:02	0.001455	0.001587
106	99	**11:01**	**44:02**	**01:02**	**12:01**	**03:01**	0.001455	0.001526
107	72	32:01	08:01	07:01	03:01	02:01	0.001453	0.00194
108	138	01:01	51:01	14:02	13:01	06:03	0.001433	0.001126
109	146	02:01	15:01	03:03	01:01	05:01	0.001431	0.001076
110	147	30:01	13:02	06:02	07:01	02:02	0.001404	0.001074

Table 3. *The combined results for the 110 most frequent 5-locus haplotypes. The haplotypes are arranged according to their frequencies in the more recent (2018 registry data) study. The FER haplotypes are shown in* **boldface**. *The four haplotypes that fulfilled the FER criteria in the later study but not the earlier one are shown in* ***italics and boldface****, and the four that fulfilled the criteria only in the earlier study are* **underlined and in boldface.**

8.2.3 Impact of Finnish 5-locus haplotype frequencies on HSCT donor matching for Finnish patients (II)

To assess the practical consequences of the Finnish HLA landscape, analyses of actual Finnish registry donor searches were performed. Of the 989 unrelated registry donor transplants performed in Finland from 2003 to 2016, 78 % were 10/10 matched (unpublished). Every year there have also been patients who have either not been transplanted at all, or, in recent years, have received a haploidentical transplant because no suitably HLA matched registry donor has been found. The proportion of these patients has been approximately 5 % (unpublished). It follows that a 10/10 matched donor has been found for a little less than three quarters of Finnish patients. In study II, a shorter period was analyzed, but all registry searches (n=647) that had resulted in confirmatory typing of donors were analyzed, regardless of whether the patient had been transplanted or not. The overall results were consistent with the unpublished data: of the actual URD transplants, 76 % had been 10/10 matched, whereas for 74 % of patients at least one 10/10 matched unrelated donor had been confirmatory typed. Thus, all confirmatory typed donors had been mismatched for 26 % of patients. The patients whose search results had been so meagre that no donors had been chosen for confirmatory typing could not be included, as the donor supplies change over time and the erstwhile results are not saved.

When the search results were divided according to the patient's probability of carrying a FER haplotype, significant differences between the groups were apparent. As expected from the estimated haplotype frequencies, 25 % of the patients carried at least one FER haplotype. These patients were less likely to have found a single 10/10 matched donor (60 % versus 79 %; $P<0.0001$), more likely to receive a mismatched transplant (34 % vs 21 %; $P=0.01$) and, when transplanted from a matched donor, more likely to receive the transplant from a domestic donor (46 % vs 18 %; $P<0.001$). The results show that in spite of the large European and North American registries, also donors with a specifically Finnish background are needed, and a higher number of such donors would likely be beneficial for Finnish patients.

8.2.4 Impact of Finnish haplotype frequencies on a registry donor's probability of being chosen to donate (II)

The HLA phenotypes of Finnish donors chosen to donate either for Finnish (n= 83) or for foreign (n=40) patients were analyzed and the donors divided into probable FER-haplotype carriers and non-FER-haplotype carriers, according to the estimated Finnish haplotype frequencies. Compared to Finnish patients or the average Finnish registry donors, both of which are more likely to represent the average Finnish population, the donors who had actually donated their stem cells were significantly more likely to carry at least one FER-haplotype. The proportion of probable FER-haplotype carriers in the average population is 25 %, whereas it was 40 % (P=0.0054) among the domestic donations and as high as 45 % (P=0.0084) in the foreign donations. The results indicate that in addition to supplying stem cells for Finnish patients, FER haplotype positive Finnish donors are needed also outside of Finland, possibly for patients with a Finnish background.

8.3 EXTENDED HLA HAPLOTYPES IN FINLAND (III)

8.3.1 HLA-DPB1-associations of Finnish 5-locus HLA haplotypes

The annual number of newly recruited stem cell donors in Finland has recently varied from approximately 3 000 to 10 000, and, since 2015, the donors have been HLA typed by sequencing techniques for the six classical HLA gene loci at the time of recruitment. This has rapidly increased the number of high resolution typed donors in the registry, and made an updated haplotype frequency analysis of the Finnish population possible. In addition to the traditional 5-locus haplotype frequencies, also the 6-locus frequencies could now be estimated, which enabled an analysis of the DPB1 associations of the 5-locus HLA haplotypes. Because of the wide variation of HLA alleles and haplotypes, a more detailed study of these associations was performed for the 100 most frequent 5-locus haplotypes only. Contrary to the conception of a weak linkage disequilibrium between HLA-DPB1 and the other class II HLA loci, Finnish 5-locus HLA haplotypes were found to carry widely divergent

and statistically significant DPB1 associations. The associations were more conspicuous among the FER haplotypes: of 34 FER haplotypes, all carried at least one statistically significant DPB1 association, while of the non-FER haplotypes, 19 of 66 did not (p=0.0002). While DPB1*04:01 allele is by far the most frequent DPB1 allele in Finland (f=0,40) as well as in other European countries, it was the most frequent DPB1 allele for only 50 % of the hundred analyzed 5-locus haplotypes. DPB1*04:02 (f=0,20) was the most frequent DPB1 allele for 15 % of haplotypes, and the respective numbers for the rest of the most frequent DPB1 alleles were: DPB1*02:01 (f=0.14) 11 %, DPB1*03:01 (f=0.12) 13%, DPB1*01:01 (f=0.05) 4 %, DPB1*05:01 (F=0.02) 2 %, DPB1*14:01 (f=0.01) 2 % and DPB1*15:01, 16:01 and 19:01 (f<0.01) 1%. The proportion of the most frequent haplotype-specific DPB1 allele varied from 29 % to 100 %, with the median for all haplotypes being 71 %. Again, FER-haplotypes differed from the others: the median for FER haplotypes was 81 % (range 40 %-96 %) while it was 62 % (range 29 %-100 %) for the non-FER haplotypes (Figure 9). The results indicate that DPB1 associations of 5-locus haplotypes are not random, and that predicting the DPB1 matching probabilities of unrelated donors is feasible.

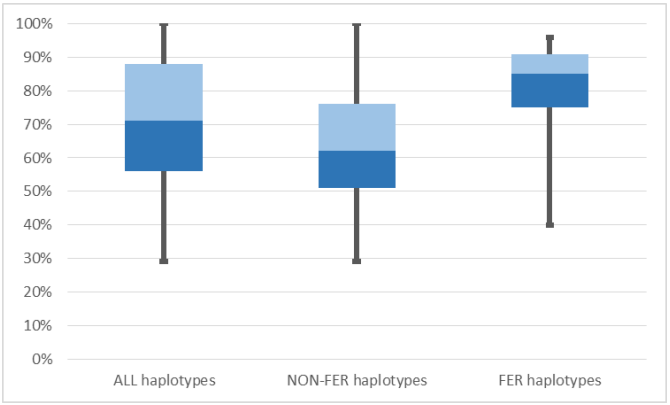

Figure 9. *Medians and ranges for the proportion of the most frequent DPB1 allele for individual 5-locus HLA haplotypes. All the hundred most frequent Finnish 5-locus haplotypes on the left, the non-FER haplotypes (n=66) in the middle and the FER haplotypes (n=34) on the right.*

8.3.2 Impact of DPB1-associations on DPB1 matching in Finnish unrelated donor stem cell transplants

Of the 769 10/10 matched transplants that had been performed in Finland between 2003-2016, 235 (31 %) had been from a domestic and 534 (69 %) from a foreign donor. Of domestic donor transplants 97 (41 %) had been matched for both DPB1 alleles, while the number was 133 (25 %, $p<0.0001$) for transplants from a foreign donor. In the study, the DPB1 mismatched transplants were further divided into 11/12 (one DPB1 mismatch) and 10/12 (two DPB1 mismatches) transplants. 122 (52 %) of Finnish donors had been mismatched for one DPB1 allele, and only 16 (7 %) for two DPB1 alleles, whereas for foreign donors the respective numbers were 309 (58 %, $p=0.13$) and 92 (17 %, $p<0.0001$). The three Finnish transplant centers have for years had different donor selection practices, with one center (TC1) prioritizing DPB1 matched registry donors and the other two (TC2 and TC3) prioritizing other donor characteristics over DPB1 match. This led us to analyze the proportions of DPB1 mismatches separately for the centers. The transplant center with DPB1 priority, had achieved a total number of 66 of 140 domestic transplants (47 %) being DPB1 matched, while 90 / 311 (29 %) of their foreign transplants had been DPB1 matched ($p=0.0003$). For the other two transplant centers, analyzed together, the respective numbers were 31/95 (33 %) and 43/223 (19 %), $p=0.013$. TC1 had minimized the frequency of two DPB1 mismatches as well: only three (2 %) of their 10/10 matched domestic transplants had been mismatched for both DPB1 alleles, while the number was 35 (11 %) for foreign donors, $p=0.0008$ (Figure 10). These results show that the DPB1 associations of the 5-locus haplotypes in Finland are either different from other Europeans, or that the associations in Finland are stronger. Considering the Finnish HLA landscape and the fact that most isolates show substantially higher levels of LD than outbred populations (Service *et al.*, 2006), it is in fact likely that both explanations are true.

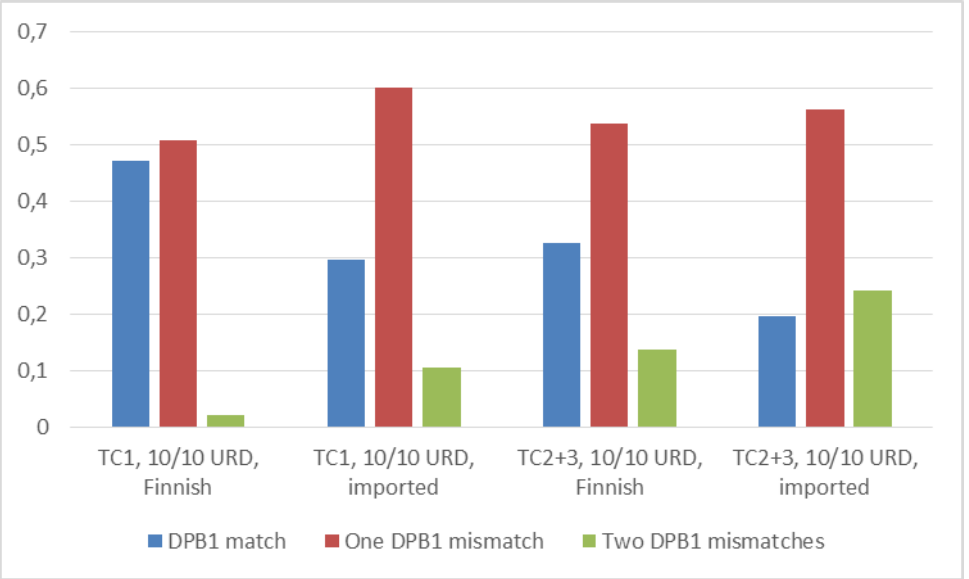

Figure 10. *The proportion of DPB1 matched and one or two DPB1 allele mismatched transplants, when the donor is either Finnish or foreign. Transplant center 1 (TC1) has prioritized DPB1 matching over other donor characteristics, while transplant centers 2 and 3 (TC2, TC3) have not.*

8.3.3 DPB1 matching in worldwide donor searches for patients with two non-FER haplotypes

To examine whether the internationally frequent HLA haplotypes carry significant DPB1 associations as well, URD searches for 84 Finnish patients, each carrying a different combination of frequent 5-locus HLA haplotypes, were performed in the BMDW database. Although the majority of registry donors in the BMDW database are not DPB1 typed, 67 of the 84 searches resulted in more than 100 DPB1 typed 10/10 matched international donors, and only one in no DPB1 typed donors worldwide. The number of Finnish donors was lower: 11 (13 %) patients had no DPB1 typed Finnish donors, and only two had more than a hundred; the median of DPB1 typed Finnish donors was 9 per search. 79 searches (94 %) yielded at least one

DPB1 matched donor. When the search results for the 84 searches were grouped together, DPB1 match probability of Finnish donors clearly differed from international donors: of the 1521 Finnish donors, 452 (30 %) were DPB1 matched with the patient, while the proportion was 1308 out of the 7407 international donors (14 %; $p<0.0001$). The proportions were higher for the 34 patients who, according to the EM analysis, carried the most probable DPB1 alleles: 372/872 (43 %) for Finnish donors, and 633/2994 (21 %) for international donors. DPB1 match frequencies were further compared between searches for patients carrying identical DPB1 alleles but different 5-locus haplotype combinations. 16 of the 84 searches were for a DPB1*04:01-homozygous patient, and for these patients, the DPB1 match frequency in international searches varied between 1/28 (3.7 %) and 55/100 (55.0 %), $p=0.0001$. For the 12 patients with allele pair DPB1*04:01, 04:02 the variation was from 4/100 (4.0 %) to 36/100 (36.0 %) $p<0.0001$. The extremes for the eight searches with allele pair DPB1*03:01,04:01 were seen in searches resulting in few 10/10 matched donors (0/7 to 4/7), but large differences were seen in more prolific searches as well (6/100 (6 %) vs 40/100 (40 %), $p<0.0001$). The widely differing DPB1 match frequencies clearly indicate that DPB1 associations of the 5-locus haplotypes are not random outside of Finland either.

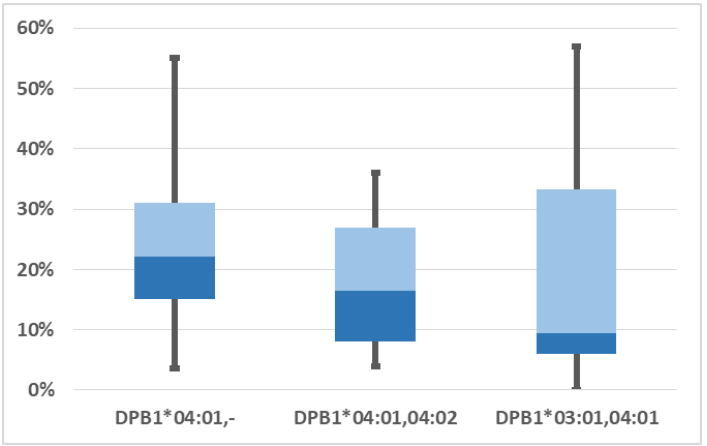

Figure 11. *Proportions of DPB1 matched international donors for 16 patients homozygous for DPB1*04:01 allele (left), 12 patients heterozygous for DPB1*04:01 and 04:02 (middle), and 8 patients heterozygous for DPB1*03:01 and 04:01 (right). Each patient carries a different 5-locus haplotype combination.*

8.4 SELECTING HLA-ACCEPTABLE PLATELETS FOR HLA IMMUNIZED PATIENTS (IV)

8.4.1 Predictors for inferior platelet transfusion responses in HLA immunized patients

The majority of the 40 patients included in the study were highly HLA immunized: 35/40 (88%) had a PRA% ≥ 80%, and 25/40 (63%) ≥ 95-100%; the median of the PRA values was 96%. Of the 270 analyzed transfusions, 37 were from two donors against whom the patient carried widely differing levels of donor specific antibodies. These transfusions were excluded from further analyses. For the rest of the transfusions, in both multivariate analysis and the logistic regression model predicting the probability of a good transfusion response, donor specific HLA antibody levels (~−2 ACI/1000 MFI, P <0.001) and ongoing infection (−9,9 ACI, P =0.004) played a significant role as independent risk factors for inferior transfusion responses (Figure 12). Cumulative MFI level of 1000 maximized the sensitivity and specificity for the

risk of inferior transfusion response in the ROC analysis, and this was confirmed by the $\chi 2$ test and Fisher's exact two-tailed test. A considerable proportion of transfusions against cumulative MFI levels above 1000 MFI were acceptable, but when the cumulative levels were higher than 5000 MFI (n= 20), acceptable transfusion responses were infrequent (21 %) (Table 4). Also the number of individual donor specific antibodies had a significant impact (−7,8 ACI/additional antibody, P <0.01), and it displayed a statistically significant interaction with the HLA antibody levels (p<0.05). The age of the infused platelet product had a significant impact (-2,6 ACI/ one day of age, p=0.04) in multivariate analyses, but not in the logistic regression model.

	ACI (18-24h) 10^9/l median (range)	ACI (18-24h) >15x10^9 % of transfusions	No of transfused platelets x $10^{11}/m^2$	Number of transfusions
HLA matched	36 (-61-103)	86 %	2,6 (1,5-3,7)	50
No DSA	34 (-11-123)	76 %	2,7 (1,9-3,5)	45
DSA 200-1000 MFI	25 (-11-92)	72 %	2,6 (2,1-3,9)	35
DSA 1000-5000 MFI	21 (-28-88)	54 %	2,6 (1,6-3,7)	86
DSA > 5000 MFI	4 (-15-34)	21 %	2,5 (1,8-3,7)	20

Table 4. *The proportions of acceptable platelet transfusion responses against different levels of cumulative donor specific antibodies.*

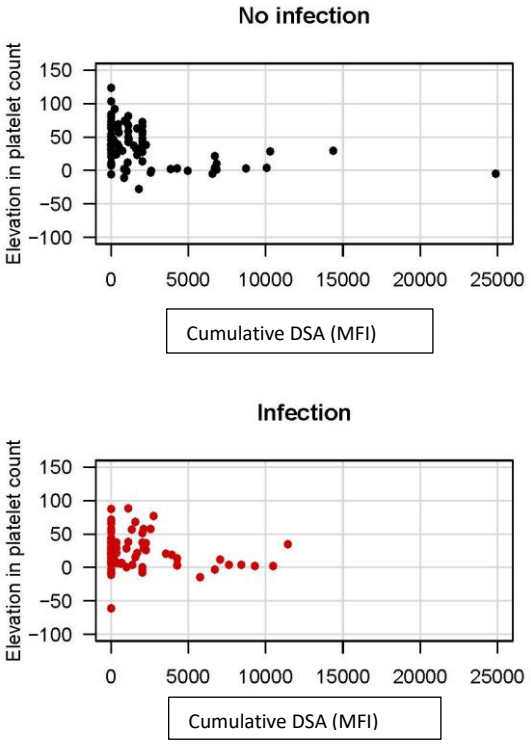

Figure 12. *Absolute platelet count increments at 18-24 h in relation to the cumulative donor specific antibody levels (MFI).*

8.4.2 Relation of HLA eplet mismatch numbers with HLA antibody levels

It would often be quicker and cheaper if platelet donors could be selected without HLA antibody testing, using the relative similarity of the donor's and patient's HLA antigens as the sole selection criterion. For this, an algorithm called Matchmaker can be used (Duquesnoy, 2006, 2008). It counts the number of mismatching eplets between the patient's and donor's HLA-A and –B antigens. To evaluate the predictive value of the eplet mismatch number for the presence of HLA antibodies in our material, the levels of all HLA antibody specificities and the number of eplet mismatches for the respective antigens were analyzed for all patients. The results are depicted in Figure 13.

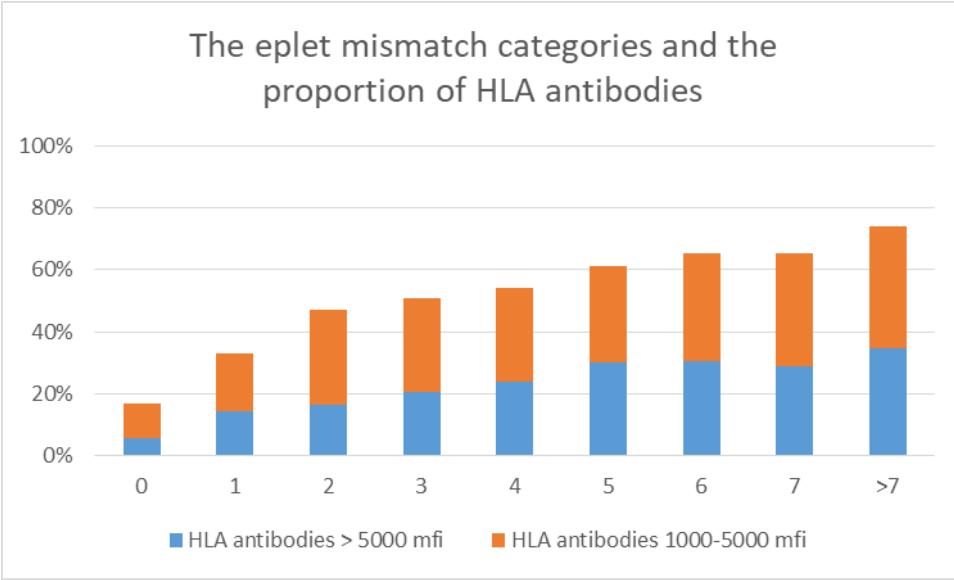

Figure 13. *Comparison between the number of eplet mismatches and the proportion of antibody positive HLA antigens.*

Any antibody with a reactivity stronger than 1000 MFI was deemed positive, and antibodies with strengths higher than 5000 MFI as strongly positive. The eplet mismatch number did carry an association with the probability of HLA antibody positivity: the likelihood was higher for HLA antigens with higher numbers of eplet mismatches, but HLA antibodies against antigens with only 0 or 1 eplet mismatches were quite frequent as well (16% and 33% respectively). High antibody levels (> 5000 MFI) represented 32-49% of all antibodies, and also high level antibodies were seen against antigens with few eplet mismatches. The only HLA antigens against which the patients never carried HLA antibodies were the patient's own. On the other hand, a considerable proportion (26 %) of antigens with more than seven eplet mismatches were antibody negative. 59% of antibody negative non-self HLA antigens were mismatched for at least 4 eplets (Figure 14). Thus, although an association between the number of eplet mismatches and the existence of a respective HLA antibody unquestionably existed, it was not strong enough for optimally

predicting the acceptable HLA mismatches for an individual, highly HLA immunized patient.

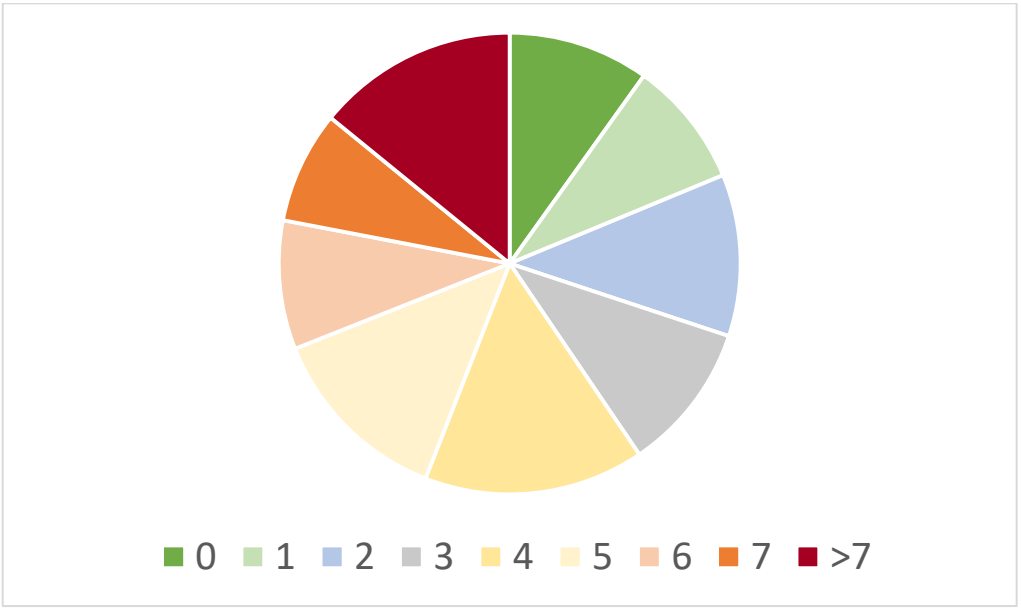

Figure 14. *HLA antigens with 0 to >7 eplet mismatches and their proportions of all antibody negative HLA antigens. A significant proportion (59 %) of antibody negative non-self HLA antigens were mismatched by at least four eplets with the patient.*

8.4.3 The frequency of HLA immunization in platelet transfusion refractory patients

Earlier on, Finnish patients could be repeatedly transfused with HLA selected platelets, based solely on a clinical suspicion of immune mediated refractoriness. To assess the justification of this policy, we analyzed a separate group of HLA antibody analyses performed for hematological patients. 108 patients (55 men and 53 women)

had been analyzed for HLA antibodies due to platelet transfusion refractoriness between 2013 and 2016 in the FRCBS HLA laboratory. The frequency of HLA immunization, high enough to explain the platelet transfusion refractoriness, was retrospectively assessed for this group. A total of 47 patients (44 %) were with PRA % ≤1%, a result not even partly explaining the refractoriness; of this group 32 (68 %) were men and 15 (32 %) women (P =0.002). Men were also less likely to be highly immunized than women: only 14 (25%) of men had PRA% higher than 50%, whereas for women the number was 33 (62%) (P =0.0002). The results likely reflect the central role of pregnancies in clinically significant HLA immunization, and, on the other hand, raise questions as to the reasons of platelet transfusion refractoriness in men. They also highlight the utility of HLA antibody testing before sustained transfusion support using HLA selected products, which is both costly and likely to cause delays in therapy.

9 DISCUSSION

9.1 THE FINNISH HLA ISOLATE

I can easily recall the registry donor search that initiated my interest in the Finnish HLA haplotypes. The search in the worldwide database, for this elderly Finnish patient, resulted in hundreds of HLA-A, -B, -DRB1 matched international donors, but virtually all were HLA-C- mismatched. At the end of the day, five HLA-C-matched donors were however detected on the list. One of them was from Switzerland, one from Sweden and three from Finland; in fact all HLA-C-typed Finnish donors seemed to be HLA-matched with the patient. At the time of this search, serological HLA-C-typing results were not shown in the international search lists, but when an additional search was performed in the domestic registry database, fifteen additional Finnish donors, all serologically HLA-C-typed, were found, and all except one carried the same exceptional HLA-C allele as the patient. The difference between Finnish and foreign donors was so obvious and so surprising that it left a permanent mark. When the first high resolution haplotype frequency estimates by David Steiner (Steiner Ltd.) from the Finnish registry database (unpublished) became available in 2013, it was evident that the referred haplotype was not by far the only one that was peculiar to Finland. Our finding that, on the other hand, two thirds of the Finnish HLA haplotypes are frequent among other Europeans as well, and one third is specifically enriched in Finland, probably reflect the somewhat dualistic character of the Finnish pedigree: it has been reported to be mostly European but to contain some Eastern/Uralic components as well (Guglielmino *et al.*, 1990; Zerjal *et al.*, 1997; Salmela *et al.*, 2008). The very sparse population in the unfavorable climate conditions has led to repeated bottleneck events during centuries, and this has probably taken care of the rest. The Finnish HLA heritage has had a profound impact on the Finnish Stem Cell Registry. In the 90's, when international registries were

small compared to the present situation, it was easiest to procure HLA matched donors from the domestic registry. Even now, when the German registry alone has more donors than Finland has inhabitants, there are several Finnish patients annually, whose only HLA matched donors are Finnish. This, together with the high homogeneity of the Finnish HLA, has led to a relatively high usage of FSCR, compared to most of the small to medium sized European registries. The fact that Finnish HLA haplotypes seem to significantly extend beyond the traditional 5-locus haplotypes has also enabled the Finnish transplant centers to avoid DPB1 mismatching, and thus lower the risk of aGvHD, years before the international registries began to DPB1 genotype the newly recruited donors upfront.

9.2 FROM THEORY TO PRACTICE

As the motivation behind the separate studies of this thesis has been very practical, also the results of each individual study have been readily implementable to the histocompatibility testing and URD search practices, and have, by now, all been put to use in the FCRBS.

After the LOH study, the validity of a potential HSCT patient's homozygous HLA typing results is now always ascertained in the FCRBS HLA laboratory by several simple methods: the patient's diagnosis, the frequency of the resulting HLA haplotypes in the respective population, the family typing results, and the presence of background reactivity in the typing analysis are always confirmed. If any suspicion of an erroneous result remain after these check-ups, the analysis is repeated by a second, more sensitive typing method, or an extra sample is requested from the treating hospital.

The study on the impact of HLA antibody levels on transfusion responses was initiated because two different cut-off levels had been in use in the FRCBS but no

studies could be found to back up either. Our study indicated that both our former levels (5000 MFI and 1500 MFI) had been too high, and the cut-off was accordingly further lowered down to 1000 MFI. Also the observed deleterious impact of multiple individual DSAs is now considered: antigens with lower (500-999) MFI levels are not applied when more optimal platelet donors are available, and whenever platelet donors carrying DSA positive HLA antigens have to be accepted due to a patient's extensive immunization, only one DSA positive HLA antigen at a time is allowed. As the predictive value of the HLA Matchmaker system for the respective antibody levels seemed relatively low, and almost half of the platelet refractory patients on the other hand do not carry significant HLA antibodies, the urgency of HLA antibody samples is emphasized to the treating physicians, and the period of providing HLAMatchmaker-based platelet products is thus kept as short as possible.

The primary idea behind assessing the Finnish 5-haplotype frequencies was to be able to predict the 5-locus high resolution typing result of both patients and donors when only the first, low resolution typing result of 3 loci is available. Accurate predictions save both time and costs, when the donor candidates for confirmatory typing must be selected out of several low resolution typed donors. The Finnish HLA landscape was shown to be unique compared to other European populations, in regard to both homogeneity and HLA haplotype frequencies. These characteristics were utilized when the Finnish registry donors were used as a pilot population for registry specific matching predictions in the WMDA Search and Match –program. Similar registry specific predictions are now available to all partly high resolution typed registry populations, which significantly enhances the accuracy of optimal donor selection worldwide.

In addition to the registry searches, HLA genotype predictions were performed daily when Sanger sequencing was used for high resolution typing: an extra sequencing round could be avoided if the predicted allele ambiguities could be excluded concomitantly with the basic sequencing. Before the estimations for Finnish-specific high resolution haplotype frequencies were completed, US Caucasian HLA

frequencies were applied also for Finnish patients and donors, and regularly resulted in erroneous predictions.

Although NGS typing and the WMDA Search and Match registry-specific match prediction algorithms have eliminated the need to utilize haplotype frequencies for some of the previous purposes, the haplotype frequencies are still an essential tool in an HLA laboratory. They are needed for the evaluation of donor specific antibodies and the plausibility of HLA typing results, when the possibility of a typing error is weighted due to rare alleles or haplotypes, or a patient's homozygous typing result. One possible warning sign of LOH is that the patient appears homozygous for a very rare HLA haplotype, making it more improbable that both of the patient's parents have carried the same haplotype, or, in case of erroneous homozygosity in fewer HLA genes, that the resulting HLA haplotypes are very rare or have no frequency at all in the patient's population. Without detailed knowledge of the haplotype frequencies in different populations this tool would be lacking while screening potential cases of HLA LOH.

With the increase of haploidentical stem cell transplantations, also the need for the analysis of HLA haplotype segregation in families has increased, establishing yet another application for population haplotype frequencies: when a sibling is considered as a potential haploidentical stem cell donor but all four HLA haplotypes of the family are not known, the risk of an erroneous interpretation of haploidentity is highest if the seemingly shared haplotype is very frequent in the population.

An international prediction tool for DPB1 is still lacking in the worldwide registry donor database. Because of this, the Finnish 6-locus haplotype frequencies are now used in the Finnish Stem Cell Registry to predict the likelihood of a DPB1 match or the permissiveness of a DPB1 mismatch in domestic donor searches. This is essential, as the vast majority of Finnish registry donors have been recruited at a time when DPB1 was not typed upfront.

9.3 A MOVING TARGET – WHAT NEXT?

The hematological stem cell transplantation has been a field of active research since its beginning, and the pace is, if anything, speeding up. The same applies to HLA typing and antibody testing and their application in the transplant field; new typing techniques and new search programs have been introduced into widespread use even in the last few years.

Both the real time –PCR technique and next generation sequencing have been validated and implemented in the FRCBS HLA laboratory after the Loss of heterozygosity study was published in 2016. The sensitivity of these techniques in detecting the lost allele or haplotype could thus not yet be studied at the time. However, since LOH as a real, recurring phenomenon is now well recognized, both of these new typing techniques have been tested also as to this aspect. In an earlier LOH case, RT-PCR was estimated to be at least as sensitive as SSP, the technique that was shown to be the most sensitive one of our original three. In a recent, quite severe case of LOH in an AML patient, next generation sequencing was deemed to be even more sensitive than RT-PCR: a heterozygous typing result was assigned without any adjustment of cut-off levels. However, as LOH is not a frequent phenomenon, and may affect only some individual HLA genes, extensive experience as to the differences in sensitiveness between different techniques on the various HLA genes and alleles is difficult to achieve. New NGS platforms for typing more HLA genes are being developed, and some of these are already known to decrease the sensitivity for background signals (Timo Saarinen, FRCBS histocompatibility laboratory, personal communication). In this regard, co-operation between HLA laboratories might be useful, considering the increasing number of platforms that are currently used for HLA typing.

After the study on acceptable cut-off level of HLA antibodies had been published, the antibody levels in a serum of an individual platelet refractory hematological patient were found to vary significantly in repeated analyses. There had been a report of a so called pro-zone effect (Schnaidt *et al.*, 2011), which is particularly prone to affect sera with very high antibody levels, making them appear too low or even negative. EDTA pretreatment has been reported to remove the pro-zone effect (Schnaidt *et al.*, 2011). These results have more recently been confirmed by later studies (Anani, Zeevi and Lunz, 2016; Wang *et al.*, 2017; Guidicelli *et al.*, 2018). Based on these reports and the experience with the one individual patient, sera of highly immunized patients were re-analyzed with and without EDTA pretreatment in the FRCBS HLA laboratory. The HLA antibody levels of a considerable proportion of hematological patients were seen to be affected by the treatment, whereas for patients waiting for a solid organ transplantation a significant effect was infrequent. Based on these findings, EDTA pre-treatment is now applied to all sera that are analyzed for HLA antibodies in our laboratory. The significance of even very low levels (1000-2000 MFI) of HLA antibodies that was seen in our platelet study may thus at least partly result from the pro-zone effect, as EDTA pre-treatment was not yet in use at the time of the study. To assess the safety of accepting higher donor specific antibody levels, and thus increase the number of acceptable platelet donors, a new study with EDTA pre-treated sera would thus be required, preferentially in a prospective setting.

Because of the polymorphism of the HLA system, the HLA haplotype frequency estimations made from a population sample, even a relatively large one like a stem cell registry, does not give an exact picture of the whole population. The first published study on the Finnish high resolution haplotype frequencies (Haimila 2013) was done from a sample of 504 high resolution typed patients, the second study in this thesis from a registry donor sample of 9774 individuals (II). In the third study of this thesis typing results of 43 365 registry donors, of which 15 288 were high resolution typed for all six HLA genes, were included (III). Only the three most frequent HLA haplotypes had the same ranking in all three analyses, but due to the

increasing sample size, the differences between frequencies and ranks are already much less obvious between the last two studies.

The Finnish genetic structure has been shown to vastly differ between the eastern and western parts of the country (Kerminen *et al.*, 2017). For stem cell registry recruiting purposes, it would be useful to know whether similar differences apply also for the HLA diversity, in spite of the registry members consisting mostly of relatively young people, likely to move after study and work opportunities. Unpublished 5-locus haplotype frequency analyses were performed simultaneously with the extended haplotype analyses of the whole population. For these analyses the Finnish population was divided into ten geographical areas according to their available postal codes. The areas simulated the divisions used by Kerminen *et al.* (Kerminen *et al.*, 2017). The results indicate that differences have existed and that they have not yet disappeared despite the current domestic migration. For example, the frequency of the most common Finnish HLA 5-locus haplotype, HLA-A*03:01; HLA-B*35:01; HLA-C*04:01; HLA-DRB1*01:01; HLA-DQB1*05:01, is at its peak in Northeastern Finland and lowest in the Southwestern Finland, whereas the 5-locus haplotype that is the most common one in almost all other European countries (HLA-A*01:01, HLA-B*08:01; HLA-C*07:01; HLA-DRB1*03:01, HLA-DQB1*02:01) had a frequency gradient of exactly the opposite direction. The sparsely populated Northeastern Kainuu-Kuusamo region seems to have exceptionally high frequencies of several FER haplotypes: in Kainuu-Kuusamo, 10 out of 20 most frequent haplotypes were FER haplotypes, with frequencies as high as 7 fold compared to the average Finnish population. The number of registry donors living there at the time of the study (n=586) was too low for more detailed analyses, but even with these limitations, the results indicate that targeted recruitment at the area might be useful to consider.

During the study period, transplantations performed from related haploidentical stem cell donors have profoundly changed the field of hematological stem cell transplantation. Some transplant centers have ceased doing HLA-A, -B, -C or -DRB1 mismatched unrelated transplants, and their requirements for other donor

characteristics, like age, sex, HLA-DPB1 permissiveness and CMV serostatus, have increased. This challenges the stem cell registries worldwide to optimize their recruitment and HLA typing strategies, and makes understanding the population specific HLA characteristics even more important than before. NGS typing methodology is making ultrahigh resolution matching of registry donors and patients possible, possibly radically improving the transplant results when using registry donors (Mayor *et al.*, 2019). The studies on Finnish HLA characteristics (II and III) indicate that matching at such level may be more likely between Finnish patients and donors than in more heterogenic populations.

Earlier studies have reported different outcomes for HSCTs according to the ethnic backgrounds of the patients and donors, with significantly lower aGvHD in the isolated Japanese population (Oh *et al.*, 2005; Morishima *et al.*, 2013), better survival with domestic donors (Fürst *et al.*, 2013) and better results when the patient and donor are likely to carry identical 5-locus or MHC haplotypes, as opposed to identical HLA alleles distributed in different haplotype combinations (Petersdorf *et al.*, 2007; Jöris *et al.*, 2013). The results of publications 2 and 3 of this thesis, as well as the results of Clancy *et al*. (Clancy *et al.*, 2019) make the notion of studying the potential differences between clinical outcomes using domestic versus imported URD transplants for Finnish patients worth considering.

9.4 FINALLY

The Finnish system of one centralized HLA laboratory and one stem cell registry, both working in close collaboration as integral parts of a national Blood Service, gives a possibility for histocompatibility specialists to work with the whole wide scope of histocompatibility testing and HLA genetics needed in hematological stem cell transplantation: from actual HLA typing, family donor histocompatibility assessing and unrelated donor searches to registry recruiting strategies, HLA antibody testing and donor selection for alloimmunised platelet transfusion refractory patients.

This has allowed the separate studies in this thesis to address diverse aspects of HLA, discoursing various very practical problems that have been encountered during day-to-day work at an HLA laboratory serving hematological transplant centers and HSCT patients. In consequence, some of the results can also directly, and in many HLA laboratories with minimal costs, be introduced into the daily practices.

However, co-operation between HLA laboratory, stem cell registry and the hematologists who actually treat the patients is crucial for optimally utilizing the results of this kind of studies. As to HLA-LOH, although HLA laboratories can independently do several additional check-ups to ascertain correct typing results, this is not always enough. The risk of mistyping, and also the probability of unnecessary repeated HLA testing diminishes when the HLA laboratory receives information on the patient's diagnosis and the proportion of malignant cells in the sample from the treating physicians. In addition to LOH, somatic mutations resulting in an HLA null allele or a novel HLA allele are encountered in leukemia cells. When HLA homozygosity or rare HLA alleles are encountered in leukemia patients, a new typing sample either in remission or from germline cells (i.e. buccal swab) may thus be necessary, again requiring collaboration from the clinic. In regard to the platelet transfusion responses, an individual platelet transfusion response is affected by many patient and product related variables. A meticulous monitoring of transfusion responses and relating the results to the blood service enables a more accurate and safe platelet donor selection for HLA immunized patients. A close collaboration between the HLA laboratory and the patient treating hematologists can thus not be over-emphasized.

The fact that in an ordinary clinical HLA laboratory these kinds of unanswered questions have arisen is a reflection of the complexity of the HLA system. Although major leaps have been taken from the beginning of the HLA era in the 1950's, when different Class I HLA antigens were discovered using sera of alloimmunised mothers, even current knowledge may represent a tip of the iceberg. Many improvements on

how to select optimal stem cell and blood donors and thus improve patient outcomes are still on their way.

10 CONCLUSIONS

The first study evaluated the frequency, warning signs and detection methods for loss of HLA heterozygosity when a leukemia patient with active disease is being HLA genotyped. The different typing methods have a widely divergent sensitivity for detecting the lost allele when an imbalance between two homologous alleles is present, and it is important for an HLA laboratory to be alert for LOH and recognize the sensitivity differences in the techniques they use.

The second and third studies focused on the special characteristics of Finnish HLA, which has a significant impact on the probability of finding HLA matched registry donors for Finnish patients. Finns were shown to be a homogenous population, carrying a significant proportion of HLA haplotypes highly enriched in Finland. Patients carrying these haplotypes are more dependent on the domestic registry donors, and less likely to find 10/10 matched donors. Finns also carry extended 6-locus HLA haplotypes, which make domestic registry donors more likely to be DPB1 matched than non-Finnish donors. Extended 6-locus haplotypes are likely to exist also in other populations, although they are probably not similar to the Finnish ones.

The fourth study evaluated the significance of different levels of HLA antibodies and HLA eplet mismatches in platelet transfusion refractory hematological patients, many of which need prolonged transfusion support, especially if they go through a hematological stem cell transplant procedure. There was no single specific cut-off level for acceptable donor specific antibodies, but the likelihood of a good transfusion response began to decrease already at 1000 MFI. HLA Matchmaker program alone is not sufficient to predict the acceptable HLA antigens for highly HLA immunized patients.

11 ACKNOWLEDGMENTS

The work in this thesis was carried out at the Finnish Red Cross Blood Service in Helsinki between 2011 and 2019. It was financially supported by state research funding (EVO grants).

I would like to start by extending my heartfelt thanks to my two outstanding supervisors, Docent Matti Korhonen, MD, PhD and Satu Koskela, PhD, for all their devoted help, patience and guidance. I also want to express my gratitude to Professor Martti Syrjälä, the Chief Executive of the Finnish Red Cross Blood Service, for allowing and enabling research side by side with routine laboratory and registry work.

I am most grateful to the official reviewers of the thesis, Professor Torstein Egeland, MD, PhD, and Docent Hanna Jarva, MD, PhD, for their time, and for their valued comments and excellent suggestions for improvement. I also want to thank Professor Jukka Partanen, PhD, and Docent Jukka Kanerva, MD, PhD, for agreeing to act as members of my thesis committee at such short notice.

I would like to express my appreciation for the invaluable assistance I received from all my co-authors and collaborators. Docent Eeva Juvonen, MD, PhD, has been an unsurpassed clinical transfusion expert, always willing to share her vast expertise also in the field of allogeneic stem cell transplantation. I am also grateful to Riitta Niittyvuopio, MD, PhD, and Docent Liisa Volin, MD, PhD, for their collaboration both on scientific work and on the daily tasks concerning HSCT patients. I warmly thank Professor Outi Kuittinen, MD, PhD, Ain Kaare, MD, Johanna Rimpiläinen, MD, Marja Pyörälä, MD, PhD, Hannele Rintala, MD, and Docent Marjut Kauppila, MD, PhD, for providing the invaluable clinical data necessary for assessing the significance of laboratory findings, and Jarno Tuimala, PhD, and Jarmo Ritari, PhD, for their indispensable expertise in the statistical analyses. I am extremely grateful to Hans-Peter Eberhard, PhD, Caroline Räther, MSc, and Professor Carlheinz Müller,

MD, PhD, from the German Bone Marrow Registry. They have performed an enormous and irreplaceable task in calculating and comparing HLA haplotype frequencies, and it has always been a great pleasure to discuss scientific questions and study plans with them.

I am indebted to all my dear colleagues in the FRCBS. My co-authors and friends from the HLA laboratory, Taina Jaatinen, Jouni Lauronen, and Timo Saarinen, always ready to discuss any issue and share their knowledge; the cheerful, devoted Stem Cell Registry team Anne Arvola, Anu Mourujärvi, Sanna Leiviskä, Emmi Sipiläinen and Tuija Ahonen, and the HLA laboratory team, whose excellent work has been a prerequisite for all HLA matching and antibody analyses in this thesis. I am deeply grateful to Juha Peräsaari, also of the FRCBS team, who was always willing to help with any problem, big or small, whether it concerned HLA, IT, statistics or university bureaucracy. Without his example, this thesis might never have been written.

I would also like to offer my warmest thanks to my dear sister and oldest friend Hanna, to whom I owe more than I can ever pay; my sister Pirkko and brother Mikko, both always able to brighten even the dullest day, and my namesake and friend Tiina, with her unerring ability to put things into perspective.

Finally, I would like to express my deepest gratitude to my family. My darling joyous “identical twins” Maleena and Noomi with their 15-year age difference, and my dear son Samu with his sarcastic surface and compassionate heart – these three are the one achievement that nothing else can surpass. And last, my biggest thanks go to my husband Jarmo, the rock of my life. It is, and always has been, a wonderful gift to be able to rely on his acceptance and support in whatever task I plan to undertake.

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