Innovative approaches to the detection of HLA immune escape in leukemia relapse after allogeneic hematopoietic stem cell transplantation

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für

Biologie

an der

Universität Duisburg-Essen

vorgelegt von

Ahci Müberra

aus Recklinghausen

Datum der Abgabe April 2018

To my Family

Die der vorliegenden Arbeit zugrunde liegenden Experimente wurden am Institut für Zelltherapeutische Forschung der Universitätsklinik Essen, Universität Duisburg-Essen durchgeführt.

1. Gutachter: Prof. Dr. Katharina Fleischhauer

2. Gutachter: Prof. Dr. Ulf Dittmer

3. Gutachter: Prof. Dr. Martin Bornhäuser

Vorsitzender des Prüfungsausschusses: Prof. Dr. Stefan Westermann

Tag der mündlichen Prüfung: 12.06.2018

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List of abbreviations

A	
(a/c) GvHD	(acute/chronic) graft versus host disease
3'UTR	3’untranslated region
abl	Abelson murine leukemia viral oncogene homolog 1
AID	autoimmune disease
ALL	acute lymphoblastic leukemia
allo-BMT	allogeneic bone marrow transplantation
allo-HSCT	allogeneic hematopoietic stem cell transplantation
AML	acute myeloid leukemia
anti-CTLA-4	anti-cytotoxic T-lymphocyte-associated protein-4
anti-PD1	anti-programmed cell death protein-1
ASHI	American Society for Histocompatibility and Immunogenetics
ATG	anti-thymocyte globulin
ATP	adenosine-triphosphate
auto-HSCT	autologous hematopoietic stem cell transplantation
Aza	azacitidine
B	
β2m	β2-microglobulin
bcl-2	B cell lymphoma 2 (apoptosis regulator)
BLCL	B lymphoblastoid cell line
BM	bone marrow
BMF	bone marrow failure
BMT	bone marrow transplantation
bp	base pair
BU	busulfan
C	
C(I/II)TA	class(I/II)-specific trans-activators
CAR T cell	chimeric antigen receptor T cell
CLIP	class II invariant chain peptide
CLL	chronic lymphoid leukemia
CML	chronic myeloid leukemia
CMV	cytomegalovirus
CNV	copy number variation
CPI	checkpoint inhibitors
CR (1/2)	complete remission (1/2)
CRS	cytokine release syndrome
CSA	cyclosporine A
Ct	cycle threshold
Cy	cyclophosphamide
CYT	cytoplasmic

D	
DC	dendritic cell
ddPCR	digital droplet PCR
DEK/CAN	fusion gene t(6;9)(p23;q34)
DKMS	Deutsche Knochenmarkspenderregister
DLI	donor lymphocyte infusion
DRST	German Registry for Stem Cell Transplantation
E	
E	efficiency
EBMT	European Society for Blood and Marrow Transplantation
ECACC	European Collection of Authenticated Cell Culture
ECP	extracorporeal photopheresis
EFI	European Federation of Immunogenetics
ER	endoplasmatic reticulum
F	
FISH	fluorescence in situ hybridization
FLU	fludarabine
FU	follow up
G	
G-CSF	granulocyte colony-stimulating factor
GvL	graft versus leukemia
H	
H-2	histocompatibility antigens
HC	hematopoietic chimerism
HD	Hodgkin Disease
HLA	human leucocyte antigen
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
HVR	hypervariable regions
I	
IDM	inherited disorders of metabolism
Ig	immunoglobulin
IHWS	International Histocompatibility Workshop
IMGT	international ImMunoGeneTics project
indel	insertion deletion
IR	immune reconstitution
IS	immune suppression
JAK	Janus kinase
K	
kb	kilobases
kDa	kilo Dalton
KIR	killer cell immunoglobulin-like receptor

L	
LD	linkage disequilibrium
LOH	loss of heterozygosity
LP	leader peptide
LSC	leukemic stem cells
M	
MA	myeloablative
mAb	monoclonal antibody
MC	mixed chimerism
MDS/MPN	myelodysplastic syndrome/myeloproliferative neoplasms
MGB	minor groove binding
mHAg	minor histocompatibility antigens
MHC	major histocompatibility complex
MHC	major histocompatibility complex
MMUD	mismatched HLA-matched unrelated donors
MRD	minimal residual disease
MSC	mesenchymal stromal cells
mTOR	mechanistic target of rapamycin kinase
MTX	methotrexate
MUD	HLA-matched unrelated donors
MW	molecular weight
N	
N	number
NFAT	nuclear factor of activated T cells
NGS	next generation sequencing
NHL	Non-Hodgkin’s Lymphoma
NK	natural killer
NMA	non-myeloablative
N-ras	neuroblastoma RAS viral oncogene homolog
NRM	non-relapse mortality
O	
OS	overall survival
OSR	Ospedale San Raffaele
P	
p53	also known as TP53; tumor protein
PB	peripheral blood
PBMC	peripheral blood mononuclear cell
PCD	plasma cell disorder
PCR	polymerase chain reaction
PID	primary immunodeficiency
PMC	persistent mixed chimerism
PML	promyelocytic leukemia protein
PT-Cy	post-transplantation cyclophosphamide

Q	
q-PCR	quantitative PCR
R	
RIC	reduced intensity conditioning
S	
SBT	sequence based typing
SNP	single nucleotide polymorphism
SSOP	sequence-specific oligonucleotide probes
SSP	sequence-specific primers
STR	short tandem repeat
T	
TAP	transporter associated with antigen processing
TBI	total body irradiation
TCR	T cell receptors
TK	thymidine kinase
TKI	tyrosine kinase inhibitors
TM	transmembrane
Tregs	regulatory T cells
TRM	transplant-related mortality
U	
UCB	umbilical cord blood
UD-HSCT	unrelated donor hematopoietic stem cell transplantation
UKE	University Hospital Essen
UPD	uniparental disomy
V	
VNTR	variable nucleotide tandem repeats
W	
WBC	white blood cell

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1. Introduction

1.1 Hematopoietic stem cell transplantation

1.1.1 Definition

Hematopoietic stem cell transplantation (HSCT) is an established treatment procedure for severe malignant or non-malignant blood disorders, where hematopoietic stem cells are transferred into a recipient with the intention of replacing the hematopoietic system that was previously abrogated by high dose chemotherapy or irradiation.

The hematopoietic stem cells (HSC) can be derived from the patients themselves (autologous HSCT or auto-HSCT) or from a healthy donor (allogeneic HSCT or allo-HSCT). The source of HSC is either the bone marrow (BM; hence the often synonymously used term bone marrow transplantation [BMT] which refers to HSCT from BM), the peripheral blood (PB) or umbilical cord blood (UCB).

1.1.2 History

Allo-HSCT was pioneered with the use of BM as stem cell source, since BM located in the large flat bones is the blood compartment where HSC are naturally most highly enriched in the human body. The first experience with allogeneic BM transplantation (allo-BMT) in humans was described in 1957 by Donnall Thomas [1] (Figure 1.1). Thomas and Ferrebee treated six patients with irradiation coupled with chemotherapy (anti-cancer drugs) and infused BM from a healthy donor. Since little was known about histocompatibility at that time, no attempt was made to match patient and donor. Consequently, none of the patients survived beyond 100 days [2]. In 1963 Donnall Thomas showed that dogs transplanted with grafts from littermates survived long-term. This observation reflected histocompatibility matching between related donors, and paralleled the discovery of the human major histocompatibility complex (MHC) described in detail in section 1.3. In 1972, Donnall Thomas reported his first successful allo-BMT for aplastic anemia (known as BM failure), followed by successful application of this treatment to patients with end-stage acute leukemia in 1975 [3,4].

At the end of the 1970's, it was recognized for the first time that the treatment success of allo-BMT for malignant blood disorder was due to a graft versus leukemia (GvL) effect mediated by the graft against residual tumor cells [5,6]. The 1980s saw the application of this treatment to patients with congenital blood disorders such as sickle cell anemia [7,8]. In 1990, Donnall Thomas and Joseph Murray (who performed the first kidney transplantation between twins) shared the Nobel Prize for their "discoveries concerning organ and cell transplantation in the treatment of human disease". This was also a visionary price, since the real clinical success story of HSCT was yet to come. In the 1990s granulocyte colony stimulating factor (G-CSF) found its way into clinical application for the expansion of HSC in the PB and the use of these PB stem cells (PBSC) for engraftment into patients [9-11]. This revolutionized the field of stem cell donation, because it not only facilitated the donation process itself, which for BM requires general anesthesia, but also greatly increased the number of obtainable stem cells, since repeated sessions of PBSC donation are possible without added clinical risk to the donor. This enabled the intention of so-called "mega-doses" of HSC necessary to partly overcome histocompatibility barriers and to allow the successful HSCT from haploidentical family donors in the late 1990s [12], an increasingly used HSCT modality as discussed below. Additional reasons for the significant rise in the clinical application of HSCT in the 1990s were the introduction of so-called "reduced intensity conditioning" (RIC) protocols for pre-transplant chemotherapy, which opened the door to treatment of elderly or less well performing patients, as well as increased accuracy of tissue typing by the advent of molecular typing methods. All this led to a constant increase in the clinical application of both auto-HSCT and allo-HSCT which continues to this day (Figure 1.1 and section 1.4). Today, allo-HSCT is the most widely used and clinically explored form of cancer immunotherapy, appointed as the Science breakthrough of the year 2013 (http://www.sciencemag.org/news/2013/12/sciences-top-10-breakthroughs-2013).

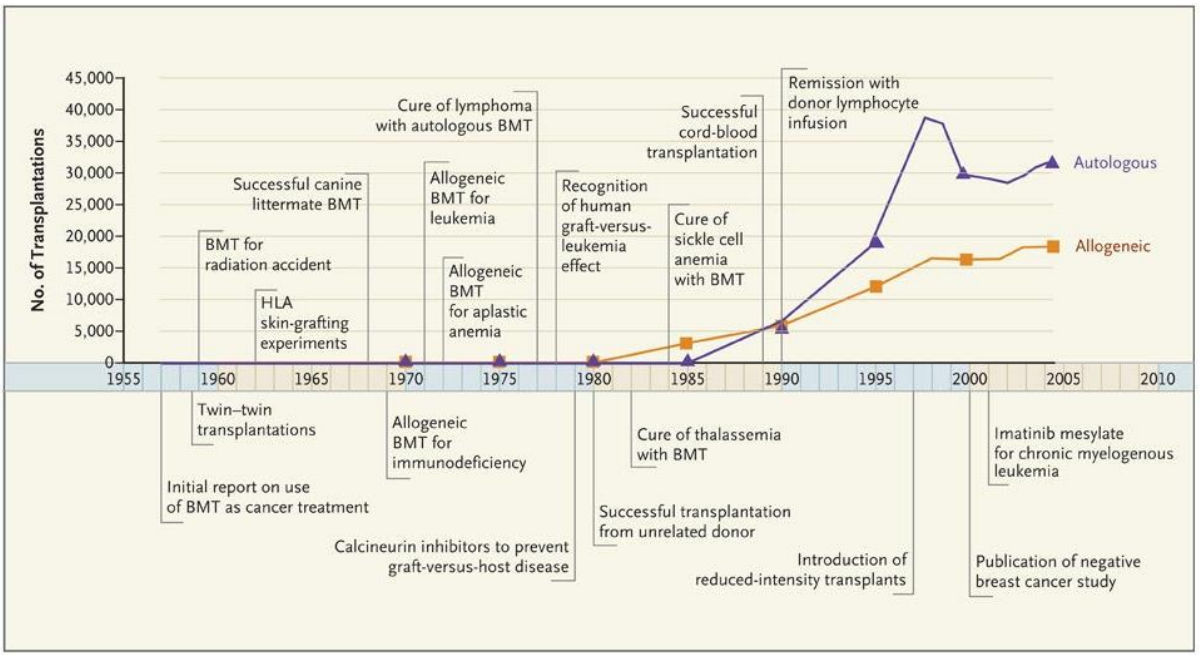


Figure 1.1: Milestones in HSCT.

Timeline showing numbers of HSCT and advances in the field from 1957 to 2006 (from Appelbaum et al. [2]).

1.1.3 Indications and HSCT performance today

HSCT is a successful treatment option for selected patients with life-threatening hematologic malignancies and non-malignant disorders. Transplant numbers are increasing unabated across the world. The European Society for Blood and Marrow Transplantation (EBMT) reported more than 42.000 transplants performed in 655 centers from 48 European countries in 2015 alone. Of these, 25.000 (59%) were autologous and 17.000 (41%) were allogeneic transplantations [13].

While the majority of allo-HSCT is performed for acute myeloid leukemia (AML), acute lymphatic leukemia (ALL), and myelodysplastic syndrome (MDS) (Figure 1.2a), as well as primary immunodeficiencies and non-malignant red cell disorders, most of the autologous transplants are performed for malignant diseases not homing to the BM, in particular so-called plasma cell disorders (PCD), i.e. multiple myeloma, as well as Hodgkin Disease (HD) and Non Hodgkin lymphoma (NHL) (Figure 1.2 b).

According to the German Registry for Stem Cell Transplantation (DRST), 3127 allo-HSCT were performed in Germany in 2016. Of these 1248 (40%) were for the cure of AML (DRST-Annual Report 2016).

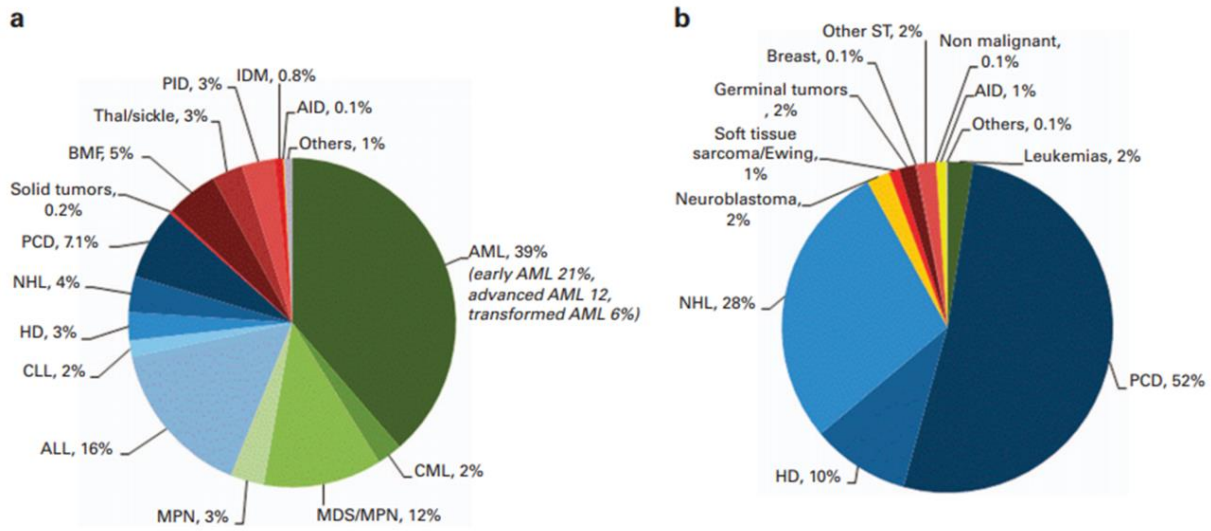


Figure 1.2: Relative proportions of main indications for HSCT in Europe in 2015.

The figure illustrates the proportions of main disease indications for (a) allogeneic and (b) autologous HSCT in Europe in 2015. AML=acute myeloid leukemia; CML=chronic myeloid leukemia; MDS/MPN=myelodysplastic syndrome/myeloproliferative neoplasms; ALL=acute lymphoid leukemia; CLL=chronic lymphoid leukemia; HD=Hodgkin Disease, NHL=Non Hodgkin lymphoma; PCD=plasma cell disorders; BMF=bone marrow failure; PID= primary immunodeficiency; IDM= inherited disorders of metabolism; AID= autoimmune disease (from Passweg et al. [13]).

The clinical success of allo-HSCT is based on the advances made over the last decades in the prevention and treatment of life-threatening infectious complications, as well as in the control of donor immune cell reactions against healthy patient tissues (graft versus host disease; GvHD), both of which determine the incidence of death not due to disease relapse (non-relapse mortality, NRM). With advances in technology and supportive patient care, HSCT has become safer, and patient survival continues to improve over time [14]. In a retrospective study, Gooley and colleagues reported a significant improvement in overall survival (OS) not preceded by relapse (disease recurrence) after allogeneic HSCT between the years 1993-1997 compared to 2003-2007 (Figure 1.3) [15]. Interestingly however, no significant advances were made in the incidence of relapse, with similar incidences of 27% and 26% in the two decades. Consistent with this, relapse is the most important cause of death after the first 100 days after allo-HSCT (Figure 1.4) [16,17]. The understanding of the biological mechanisms underlying relapse, and the development of new technologies for its early diagnosis are therefore of paramount importance and the subject of the present thesis.

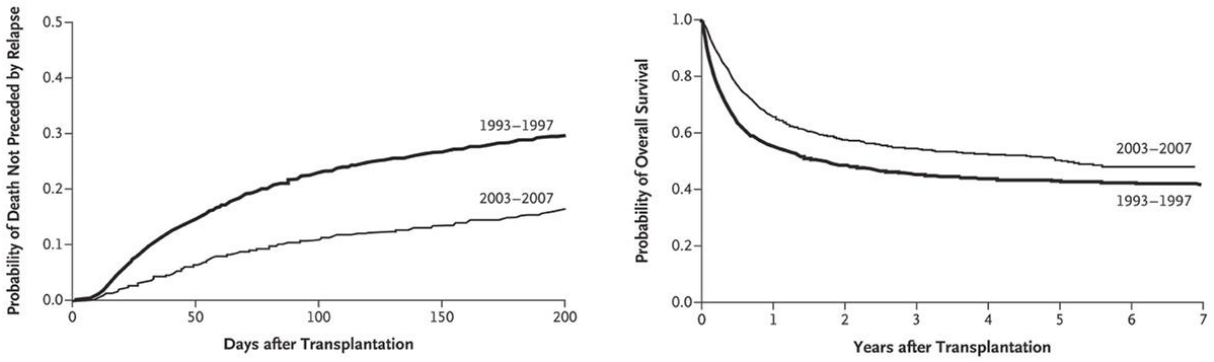


Figure 1.3: Probability of death by day 200 not preceded by relapse and of overall survival.

Panel A shows the probability of death not preceded by relapse (disease recurrence), and panel B shows the probability of overall survival during two time periods (1993-1997 [N=1418] and 2003-2007 [N=1148]). The probability of OS was estimated with the use of the Kaplan−Meier method (from Gooley et al. [15]).

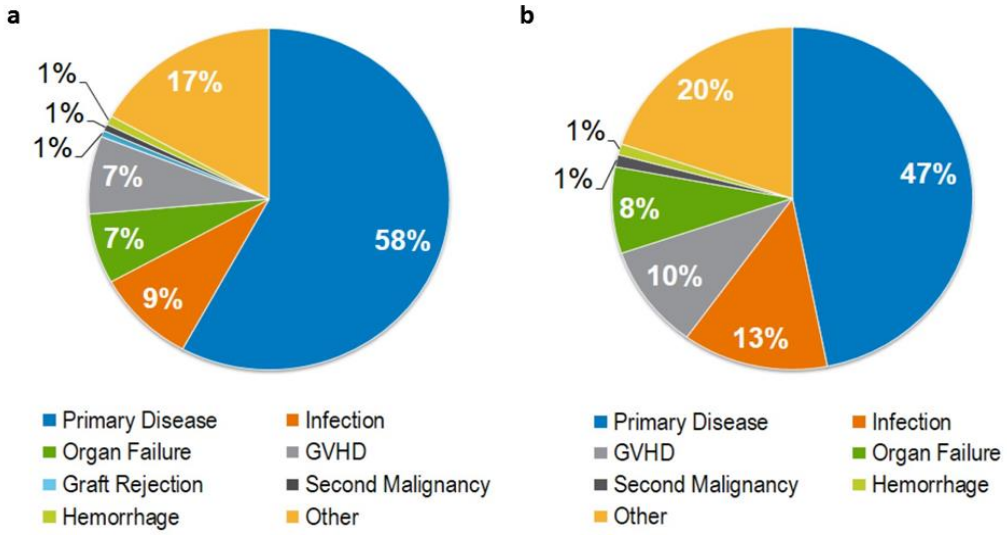


Figure 1.4: Causes of death after allo-HSCT (2014-2015).

Disease recurrence is the single leading cause for death after (A) matched sibling and (B) unrelated donor transplantation. Source: www.cibmtr.org

1.1.4 HSCT procedure

The HSCT procedure can be divided into three basic steps: patient conditioning pre-transplant, infusion of stem cells, and immune reconstitution post-transplant. **Patient conditioning** refers to the preparatory regimen needed to eliminate the patient's hematopoietic system sufficiently to make space for its replacement by the donor's healthy stem cells. This is achieved by high-dose chemotherapy with or without total body irradiation (TBI). Conditioning regimens can be either myeloablative (MA), RIC or non-myeloablative (NMA). MA regimen will ablate the patient's own hematopoiesis to an extent that autologous hematologic recovery is impossible, thereby rendering the infusion of hematopoietic stem cells from a donor (or from the patient if stored as back-up pre-transplant) mandatory for hematologic reconstitution. MA regimens are generally based on a combination of cyclophosphamide (Cy) and TBI or busulfan (BU). RIC is a special form of conditioning which has lower toxicity, often due to the replacement of Cy by fludarabine (FLU). NMA conditioning is less intense and therefore allows autologous hematologic recovery, if no allogeneic donor stem cells are infused. The introduction of RIC and NMA regimens in the 1990's has greatly widened the clinical applicability of allogeneic HSCT also to patients of older age and/or with co-morbidities. The upper patient age limit of this procedure has thereby increased from approximately 60 to over 75 years in the last decade [18-20].

The **infusion of stem cells** is performed most commonly by intravenous administration, after which they will home to the patient's BM, a process termed engraftment. While historically BM was the only HSC source (see paragraph 1.1.2), G-CSF mobilized PBSC are nowadays more frequently used than BM, due to the ease of isolation and the large number of stem cells obtainable [21]. Also UCB can be used, but the number of HSC is even more limited than with BM, requiring sometimes the simultaneous use of several UCB donors (double or triple cord blood transplantation) [22]. The choice of the graft source is dependent on different factors, in particular the patient's body weight, which is directly proportional to the number of stem cells required for engraftment. Therefore, graft sources with limited numbers of HSC such as BM and UCB are predominantly used in pediatric patients. The graft can be manipulated *in vitro* prior to infusion to reduce the number of T cells in order to avoid GvHD. However, this has the down-side of delayed immune reconstitution and higher risk of graft rejection as discussed below.

In case of blood group incompatibility between patient and donor, the graft can be purged of red cells prior to infusion. After engraftment, the donor stem cells repopulate all three blood cell lineages (i.e. platelets, white and red blood cells), a process termed **immune reconstitution (IR)**. Efficient and rapid IR is critical not only to restore a functional immune system to fight infections, but also to prevent the risk of severe bleeding due to platelet deficiency. The different lineages of white blood cells recover at a different pace, with neutrophils, monocytes and natural killer (NK) cells preceding the T and B cells of the adaptive immune system by days up to several weeks (Figure 1.5) [23]. Typically, CD8+ T cells recover more quickly than CD4+, leading to a so-called CD4+/CD8+ ratio inversion in the first months after transplantation, which converts to the normal status of CD4+ T cells outnumbering CD8+ only after several months (Figure 1.5).

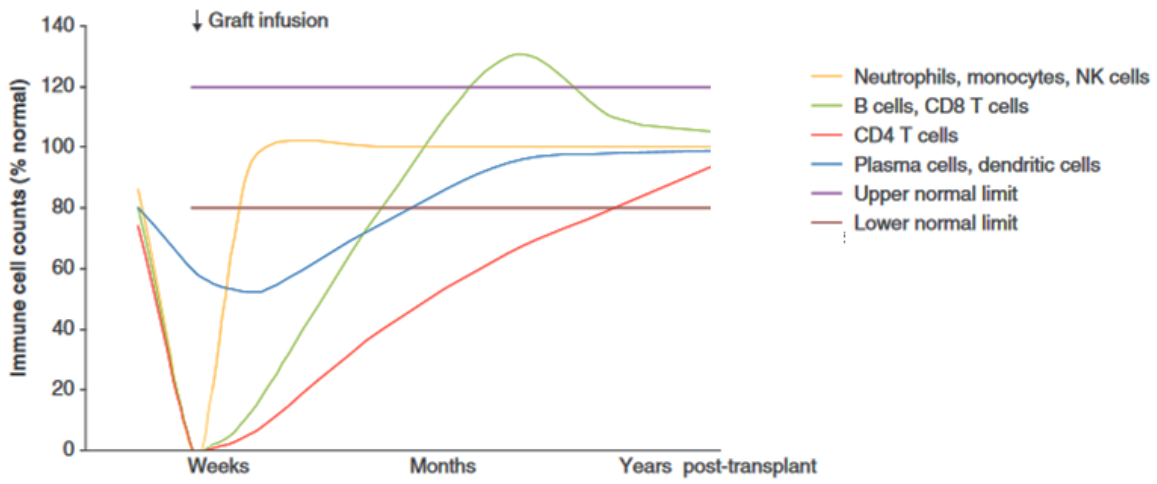


Figure 1.5: Immune reconstitution after HSCT.

Approximate immune cell counts (expressed as percentages of normal counts) peri- and post-myeloablative hematopoietic cell transplantation (from Storek [23]).

1.1.5 HSCT clinical risks

The clinical risks of HSCT can be divided into early risks (typically up to day 100 after transplantation) and late risks (100 days post-transplant). The **early risks** are mainly related to the severe status of immune deficiency induced by the conditioning regimen (see paragraph 1.1.4) and to acute GvHD (aGvHD) mediated by donor T cells recognizing major or minor histocompatibility differences between patient and donor (see section 1.3).

Immune deficiency precedes IR and exposes the patient to the risk of severe infections (viral, bacterial or fungal). Among the common post-transplant infectious complications is reactivation of cytomegalovirus (CMV), a latent herpes virus residing mainly in blood tissues of the host [24]. Depending on different factors, including T cell depletion and the pre-existing anti-CMV immunity in patient or donor, CMV reactivation occurs in up to 60% of patients after allo-HSCT and, if not controlled by antiviral therapy, can cause overt CMV disease with severe clinical symptoms and elevated NRM [25-27]. On the other hand, CMV reactivation has also been associated with reduced risk of post-transplant recurrence of AML [28-33]. This association, however, is debated and could be modulated by the use of T cell depletion [27,34-36]. The risk of infections is faced in the first month post-transplantation by total decontamination of any agent coming into contact with the patient, who is placed into a specific sterile room with inverted laminar air flow. Also the food has to be sterilized in the first weeks. The advantage of purging the gut bacterial flora by antibiotics treatment, advocated by the early work of Professor van Bekkum [37,38], has been challenged over the last years since a protective effect against aGvHD of the gut flora has been recognized [39].

aGvHD is graded into four different stages, with grades I-II generally not needing systemic treatment [40,41]. In contrast, grade III-IV aGvHD is a major and potentially lethal complication that affects mainly the patient's skin, liver and gut, and requires treatment by "heavy" immune suppression (IS) through steroids. However, some patients develop steroid refractory aGvHD, which has a poor prognosis. Available treatments of this condition include extracorporeal photopheresis (ECP) [42], as well as new drugs interfering with signaling pathways of activatory receptors on T cells, such as the JAK1/2 inhibitor Ruxolitinib [43]. In order to prevent aGvHD, immune suppressive drugs are frequently administered to the patient pre-emptively post-transplantation for several months up to years. These drugs include calcineurin inhibitors such as cyclosporine A (CSA) or tacrolimus, which interfere with the NFAT danger signaling pathway, drugs interfering with cell proliferation such as methotrexate or mofetil, and mTOR inhibitors interfering with signaling through the IL-2 receptor such as rapamycin/sirolimus. In particular in the presence of major histocompatibility mismatches with increased risk of aGvHD, the patient is often administered anti-thymocyte globulin (ATG) post-transplant. This decreases the risk of aGvHD, but interferes also with T cell IR, leading to higher risks of infection.

Moreover, graft rejection is generally not a major problem in non-T cell depleted grafts, but is more frequent in the presence of T cell depletion. Together, infectious complications and aGvHD are the major causes of transplant-related mortality (TRM), the most frequent cause of death in the first 100 days post-transplant [16].

Late risks of HSCT are represented mainly by malignant disease relapse, the major cause of death in patients after 100d post-transplant (Figure 1.4) [16,44], and chronic GvHD (cGvHD), an often invalidating complication with diffuse sclerosis of the skin and the joints [45]. Like in aGvHD, also cGvHD is graded according to its clinical extension into limited and severe, the latter being one of the most frequent causes of decreased quality of life despite cure from malignant disease through HSCT.

1.1.5 HSCT clinical benefits

HSCT is the oldest and most established form of cancer immunotherapy, with immune cells from the donor eliminating residual malignant cells from the host surviving after conditioning (GvL). This GvL effect can be mediated by different types of donor immune cells. Among the most important ones are alloreactive CD8+ and CD4+ T cells recognizing major or minor histocompatibility differences between patient and donor (see section 1.3). Since these same alloreactive T cells are often also the mediators of GvHD (see paragraph 1.1.4), the GvL effect is a double-edged sword. Considerable work has been carried out to identify strategies for separating GvL from GvHD [46]. These include the infusion of defined cellular subsets with regulatory function such as regulatory T cells (Treg) or mesenchymal stromal cells (MSC), pharmacological depletion of activated T cells early post-transplantation through the administration of post-transplant cyclophosphamide (PT-Cy), and the search for genetic mismatches leading to mild, controlled T cell alloreactivity (so-called permissive mismatches), sufficient for GvL without severe GvHD. In recent years, particular attention has been paid to new cellular mediators of selective GvL, including NK cells and $\gamma\delta$ Tcells [47]. In case of imminent or overt relapse, the GvL effect can be boosted by donor lymphocyte infusions (DLI), which however also increase the risk of aGvHD. The use of selected cell types being pioneered for increasing the safety of DLI [48].

1.2 Hemaptopoietic chimerism post transplantation

1.2.1 Definition

The term chimerism was coined from the Greek mythology of a creature hybrid between a lion, a goat and a dragon. It refers to the co-existence, in the blood of the transplanted patient, of cells from donor and from patient origin. Ideally, after MA or RIC conditioning (see paragraph 1.1.4), a so-called full donor hematopoietic chimerism (HC) should be achieved, i.e. the complete absence of cells from patient origin. However, in the NMA setting, a certain proportion of patient cells can persist post-transplantation, in a status of mixed chimerism (MC). In the MA and RIC setting, the most frequent reason for MC is disease relapse. However, MC can also be triggered by specific events such as CMV reactivation, in particular if a CMV seropositive patient is transplanted from a CMV seronegative donor [49], with cellular immunity against CMV specifically reconstituted by pre-transplant patient T cells surviving the conditioning regimen. This form of MC is also termed split chimerism, as it regards to a specific cell population with defined function. Another cause of MC can be a generalized autologous reconstitution of patient blood cells, leading to graft rejection. This form of delayed graft rejection is often preceded by a status of persistent MC (PMC), in which patient and donor cells co-exist for months to several years. PMC can lead to rejection, but can also be stable in case of established immunological tolerance, a phenomenon occurring in particular in patients transplanted for non-malignant congenital blood disorders [50].

1.2.2 Post-transplant HC monitoring

Longitudinal HC monitoring is essential in the first months after transplantation to assess the quality of engraftment and later on to integrate the analysis of minimal residual disease (MRD) by flow cytometry and, if available, the molecular tracking of tumor specific molecular markers [51,52]. While engraftment monitoring does not require high sensitivity assays, sensitivity is crucial for the application of chimerism monitoring to detect MRD.

HC monitoring is performed by the molecular detection of informative genomic polymorphisms in DNA extracted from mononuclear cells circulating in the patient's PB or BM post-transplantation.

Informative polymorphisms are those present in the patient but absent in the donor, if the purpose is to detect patient-specific cells, such as residual leukemia cells. For engraftment monitoring, also donor-specific polymorphisms (present in the donor but absent in the patient) can be used. However to achieve the double purpose of engraftment and MRD monitoring, patient-specific polymorphisms are generally preferred. The polymorphisms to be targeted can be of any kind including (but not limited to male-specific genes in case of sex-mismatched HSCT) variable nucleotide tandem repeats (VNTR), short tandem repeats (STR), single nucleotide polymporphisms (SNP) and insertion/deletion polymorphisms (indel). Also specific genes known to be mismatched between patient and donor, such as minor histocompatibility antigens (mHAg) or mismatched HLA antigens, as well as genes coding for blood groups or other polymorphic gene systems can be used.

The clinical gold standard has been the monitoring of STR, due to its technical ease and rapidity of execution. STR monitoring is based on multiplex polymerase chain reaction (PCR) amplification of different STR loci that vary by one to several base pairs in length between different individuals [53-55]. PCR products of different sizes are resolved by capillary gel electrophoresis, and the relative amount of patient and donor cells in the original sample is determined by semi-quantitative analysis of the area under the peak of patient- or donor-specific amplicons. This method was originally developed for forensic purposes and has several advantages, including a high level of standardization, robustness, and time and cost efficiency. The method has an intrinsically limited sensitivity of 1% to 5% [56-58], because of the need to keep the amount of target DNA to a minimum of “few nanograms” to avoid PCR competition and plateau biases. Limited sensitivity is not a problem for engraftment monitoring, but limits the efficacy of STR in monitoring MRD post-transplantation.

This problem has recently been overcome by quantitative real-time PCR (qPCR), a directly quantitative method evaluating the cycle threshold, which is inversely proportional to the original amount of target DNA [59]. For HC analysis, the cycle threshold (Ct) of the gene of interest is compared in reference with an internal housekeeping gene to the patient DNA pre-transplant (delta-delta Ct method). The amount of input DNA is flexible and directly proportional to the sensitivity of qPCR, which at 100 ng is more than 2-log higher than that of STR.

The first methods for qPCR-based HC determination on SNPs or indels were described over a decade ago [60-62], and several commercial kits are currently available for this purpose. The feasibility and enhanced sensitivity of this system compared with STR has been documented in different studies [56,63-66]. Most reports addressing the clinical utility of qPCR HC have focused on the endpoint disease relapse, which was shown to be detected significantly earlier by qPCR than by STR [67-69]. Consensus is still missing on the best cut-off value for positivity in qPCR as well as the preferable use of BM or PB, the latter having obvious logistical advantages for sample acquisition at sufficiently high abundance, but with potentially lower informative value compared with BM, the natural environment for relapse onset. Moreover, only a single report has addressed the question of engraftment monitoring by qPCR, in the particular setting of umbilical cord blood HSCT [70].

However, qPCR is limited by the high influence of relatively small differences in amplification efficiency on its results. Therefore, the efficiency of each SNP or indel target needs to be validated, which makes the development of qPCR assays for chimerism analysis a challenge. With the highly accurate digital droplet PCR (ddPCR), samples are partitioned into nanoliter droplets so that each droplet becomes a separate reaction chamber with 0, 1 or more copies of the target DNA molecule [71-73]. ddPCR HC quantification is performed on the mean number of positive target sequences per partition that far exceeds what is possible with the analog qPCR down to 0.01% on SNPs or indels. Since ddPCR uses end-point detection of a product, efficiency of amplification and calibration curves are not required [71]. The disadvantage of the ddPCR approach is that it is more labor-intensive and less suitable for the diagnostic routine in the clinics. Finally, next generation sequencing (NGS) has also been applied to HC monitoring [74]. This method is particularly valuable for targeting SNPs, since it obviates the above mentioned need for the design of multiple qPCR reactions under homogenous conditions. Moreover, the high throughput and accuracy of quantification by direct read-counting makes NGS a very attractive tool for HC monitoring. But its application is not recommended for targeting STRs or indels due to the bioinformatics difficulties in dissecting read shifts arising from PCR product length differences.

1.3 HLA and Histocompatibility

1.3.1 History

While the existence of red blood cell agglutinins had been known since their discovery by Karl Landsteiner in 1900 [75], the presence of an analogous antigen system on white blood cells was discovered only in the late 1950s. Jean Dausset (France), Jon van Rood (The Netherlands) and Rose Payne (USA) independently observed agglutination of white blood cells from some but not all donors by the serum of multiparous women [76-78]. This led to the description by Jean Dausset of the first human leukocyte antigen (HLA) called MAC (the initials of the three blood donors that were used to perform the experiments), later to become HLA-A2 [76]. In 1980 Dausset was awarded the Nobel Prize, together with George Snell and Baruj Benacerraf for the "discovery of the major histocompatibility complex (MHC) genes which encode cell surface protein molecules important for the immune system's distinction between self and non-self" [79,80]. Following up on these findings, Dausset, van Rood and Payne used a sophisticated computer system to dissect the specificity of 60 sera from multiparous women, thereby discovering the bi-allelic nature of the HLA genes. They soon realized that the complexity of the antigen system they were unraveling could only be comprehensively characterized through sera exchange within international collaborations. To this end, the first International Histocompatibility Workshop (IHWS) was organized in 1964, a tradition that holds to this day, with the 17th IHWS held in San Francisco in September 2017. The focus of the IHWS changed from sera exchange in the 1960s and 1970s to the collaborative design of molecular tissue typing techniques in the 1990s, and the exchange of bioinformatics data on HLA diversity obtained by NGS technology to date.

1.3.2 The Major Histocompatibility Complex

The HLA genes are encoded within the human MHC located on the short arm of chromosome 6 (6p21.3), a gene region that spans 4.000 kilobases (kb). The MHC is polygenic with more than 220 genes that fall into three classes: MHC class I and II encompassing amongst others the HLA-A, B, C and HLA-DRB, DQ and DP genes, respectively, and MHC class III consisting of genes involved in the complement cascade (Figure 1.6) [81].

Interestingly, the majority of genes in the MHC are closely involved in immunological processes, including antigen processing and immune response (see chapter 1.3.4). It is the most gene-dense and most polymorphic region in the human genome. The MHC class I region contains not only the classical HLA-A, B, C loci but also non-classical genes HLA-E, F and G, whose function in the innate and adaptive immunity is being discovered [82-85]. Within the classical HLA-DR, DQ and DP loci, the MHC class II region contains also a number of non-expressed pseudogenes (DRB2/6/78/9, DQA2, DPA2) as well as genes involved in antigen processing to HLA class I (TAP and related proteins) and HLA class II (DM, DO). A hallmark of the MHC is its enormous genetic variability, with 17,881 different genes described in the current Release 3.31.0 (2018-01) of the IMGT/HLA database [86]. Gene duplications have played an important role in the generation of MHC polymorphism which is characterized by a high degree of linkage disequilibrium (LD), i.e. the non-random association of alleles at different HLA loci [87].

The HLA alleles inherited together on the same chromosome are referred to as haplotypes. Inheritance follows Mendelian rules, with co-dominant expression of both HLA alleles from the maternal and paternal haplotype. When the two alleles at a given HLA locus are different, the HLA locus is heterozygous, when they are identical, the HLA locus is homozygous.

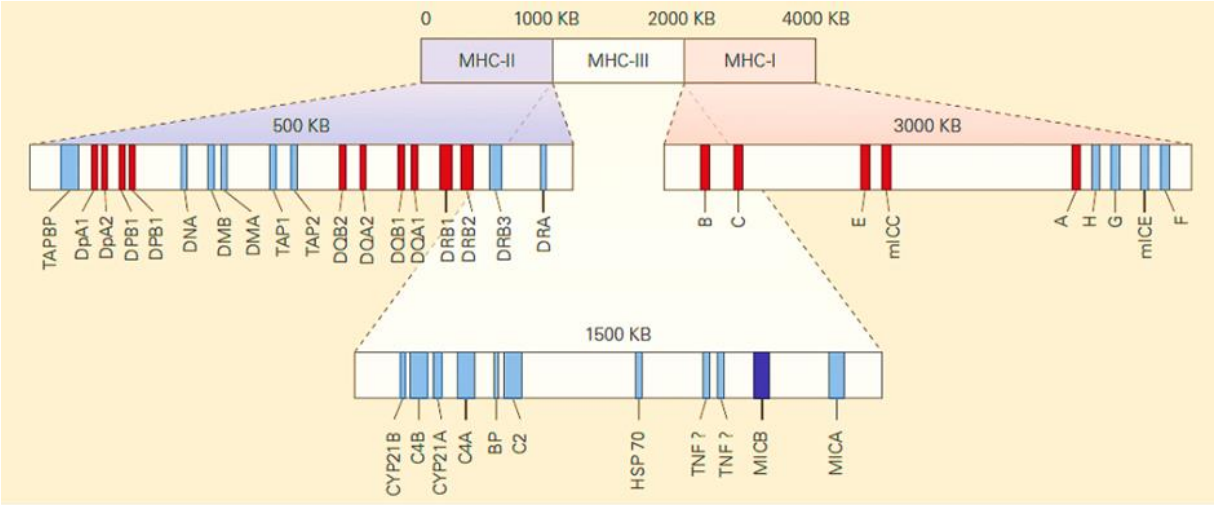


Figure 1.6: Simplified genetic map of the human MHC.

More than 200 genes are encoded within the major histocompatibility complex (MHC) class I, II and III. Main genes are indicated in red (from Stiehm [81]).

1.3.3 HLA structure

HLA molecules are cell surface heterodimers of the immunoglobulin (Ig) superfamily and as such contain several Ig domains. HLA class I molecules consist of a 45 kDa molecular weight (MW) glycoprotein (heavy chain) associated with β2-microglobulin (β2m), another Ig member of 12kDa MW (light chain). β2m is not encoded in the MHC but on human chromosome 15q and has limited polymorphism. The HLA class I coding region is 1098 bp in length and clustered into 8 different exons (Figure 1.7). Most of the nucleotide polymorphisms of the total 12,716 different HLA-A, B, C genes described to date [86] are clustered in so-called hypervariable regions (HvR) in exons 2 and 3 encoding the α1 and α2 Ig domains which form the peptide (antigen) binding groove. HLA class II molecules are heterodimers of an α and a β chain of similar size (34kDa and 30kDa MW, respectively).

The HLA class II α chain genes have a limited polymorphism with 7, 94 and 64 alleles described for DRA, DQA1 and DPA1, respectively. This is in strong contrast to the highly polymorphic HLA class II β chain genes for which a total of 4,569 different variants have been described to date for DRB, DQB1 and DPB1 [86]. The coding region of HLA class II α and β chains is 777bp in length and clustered into 5 different exons, with most polymorphisms clustered in HvR in exon 2 of the β chain.

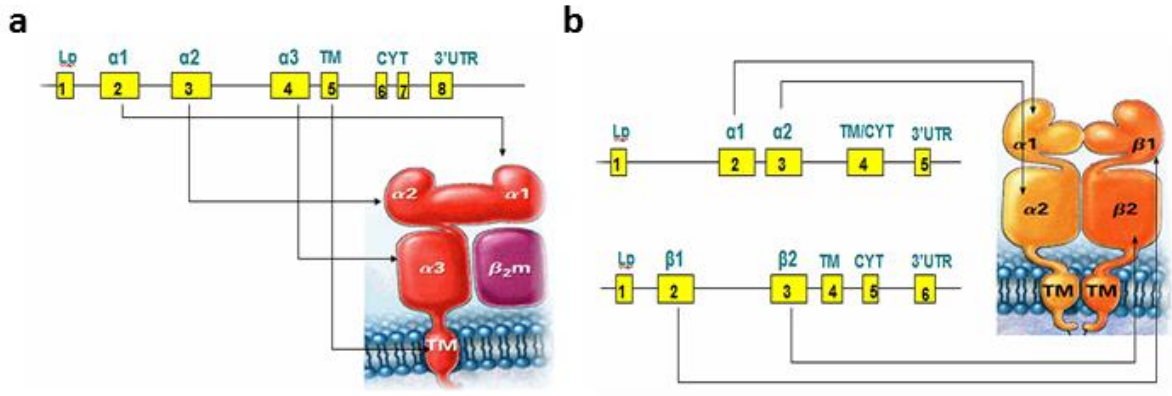


Figure 1.7: Genomic organization of HLA class I and II genes.

The image was downloaded from www.gendx.com. Shown are the 8 exons of the HLA class I heavy chain (left panel) or the 5 exons of the HLA class II α or β chain (right panel), along with the parts of the HLA molecule they encode. LP: leader peptide; TM: transmembrane; CYT: cytoplasmic; 3'UTR: 3'untranslated region.

Despite their different genomic structure and organization, the final conformation of HLA class I and II molecules is remarkably similar. They comprise overall 4 Ig domains, two of which compose the antigen binding groove, that accommodates processed peptides (see paragraph 1.3.4). The crystal structure of both HLA class I and HLA class II molecules has been resolved [88,89] and shows strikingly similar features, with peptide residues in intimate contact to polymorphic amino acids in the groove (Figure 1.8).

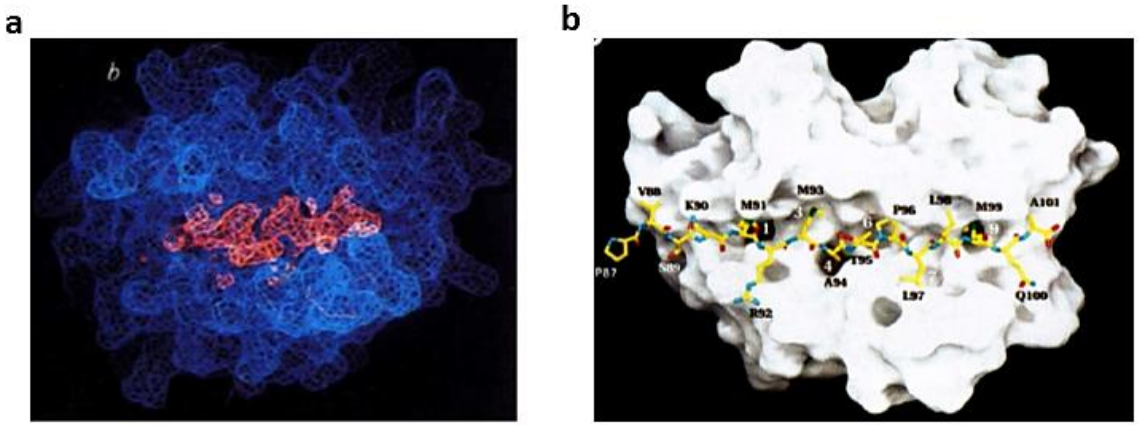


Figure 1.8: Crystal structure of HLA-A2 and HLA-DR3.

Shown is a top view of the antigen binding groove of HLA-A2 with peptide (left panel; from Bjorkman [88]) and HLA-DR3 with CLIP peptide (right panel; from Ghosh [89]).

1.3.4 HLA function

HLA class I molecules are expressed on the surface of all nucleated cells and present peptides derived mainly from intracellular proteins to the T cell receptor (TCR) of cytotoxic CD8+ T cells. Moreover, certain HLA class I molecules especially for the B and C types are additionally sensed by activating and inhibitory killer cell immunoglobulin-like receptors (KIR) on NK cells and subsets of CD8+ T cells. The generation and loading of antigenic peptides onto HLA class I molecules is a complex process [90]. Briefly, intracytoplasmic proteins are generated by proteasomal cleavage and next translocated into the endoplasmatic reticulum (ER) by an energy-dependent process involving the transporter associated with antigen processing (TAP), an MHC encoded adenosine-triphosphate (ATP)-binding cassette protein. HLA class I molecules can exit the ER only in the trimeric form composed of heavy chain, light chain and peptide.

Nascent HLA class I molecules undergo glycolytic modifications in the Golgi apparatus before they reach the cell surface. MHC turnover half-lives are variable, ranging from undetectable to a few hours, and seem to be dependent on the cell type and the HLA locus, but not the allelic variation within that locus [91]. Due to the closed shape of the antigen binding groove, peptides bound to HLA class I molecules have a restricted length of 9-10 amino acids, and are characterized by well-defined so-called anchor residues in close contact with peptide binding pockets in the groove.

HLA class II expression is in contrast restricted to certain, mainly hematologic, cell types with defined immune functions, in particular B cells, monocytes, macrophages and dendritic cells (DC), as well as activated but not resting T cells [92]. Recently, HLA class II expression has also been described on a subpopulation of neutrophils homing to the gut [93]. A number of hematologic malignancies deriving from these cell types, such as B cell leukemias and lymphomas, but also a certain fraction of myeloid leukemias do express HLA class II (see section 1.4). Restricted HLA class II expression is the result of transcriptional regulation by the class II MHC trans activator (CIITA), which co-regulates not only the classical HLA-DR, DQ and DP genes but also the non-classical HLA-DM and DOA (but not DOB) genes involved in peptide processing [94,95]. Briefly, peptides loaded onto HLA class II molecules are derived mainly from extracellular proteins that are taken up by endocytosis or phagocytosis, cleaved by cathepsins in the lysosomal compartment and then translocated into the endosomal compartment dedicated to class II loading (MIIC).

Nascent HLA class II molecules leave the ER as a nonameric complex with the Invariant Chain, which is cleaved in the MIIC to leave the minimal class II invariant chain peptide (CLIP) bound to the groove. CLIP is removed with the help of the chaperone HLA-DM, which favors binding of high affinity exogenous peptides. In certain cell types such as B cells and DC, the action of HLA-DM is antagonized by HLA-DO, broadening the repertoire of class II presented peptides [96]. Due to the relaxed conformation of the HLA class II antigen binding groove, peptides are generally longer than for HLA class I, varying between 12 and 20 amino acids, and have less well defined anchor residues. HLA class II molecules are recognized by the TCR of CD4+ T cells, thereby triggering helper, regulatory and also in some cases cytotoxic functions. Unlike for HLA class I, receptors for HLA class II have not been described on NK cells to date.

The main function of HLA class I and II molecules is the orchestration of the adaptive T cell response to pathogens and tumor antigens. During thymic development, only T cells able to recognize peptide presented by self-HLA molecules are allowed to survive (positive selection). This process results in the phenomenon of self-HLA restriction, discovered by Zinkernagel and Doherty in 1974 [97], a fundamental finding in immunology for which they were awarded the Noble Price for Physiology and Medicine in 1996. The TCR repertoire is further shaped in the thymus by negative selection, a process during which T cells bearing TCR with very high affinity to peptides are eliminated. Together, positive and negative selection ensure the presence of efficient adaptive immunity to exogenous, reducing low the chances of autoimmunity.

However, HLA molecules also mediate alloreactivity, a process by which non-self HLA is recognized by the cellular and/or humoral immune system (Figure 1.9). T cell alloreactivity is based on molecular mimicry with self-HLA restricted T cells cross-recognizing mostly self peptides bound to foreign HLA antigens (direct alloreactivity). Moreover, polymorphic peptides derived from the foreign HLA antigens can also be processed, presented in the groove of self-HLA molecules and recognized by self-HLA restricted alloreactive T cells (indirect alloreactivity). Finally, alloreactivity can also be mediated by T cells recognizing peptides derived from non-HLA polymorphic proteins encoded anywhere in the genome and presented in the groove of self-HLA molecules. These so-called minor histocompatibility antigens (miHA) are the only sources of alloantigens in completely HLA-matched transplantation [98]. Humoral alloreactivity is mediated by antibodies recognizing polymorphic epitopes on non-self HLA molecules. These alloantibodies play a major role in solid organ transplantation and in partially HLA matched HSCT (see paragraph 1.3.6).

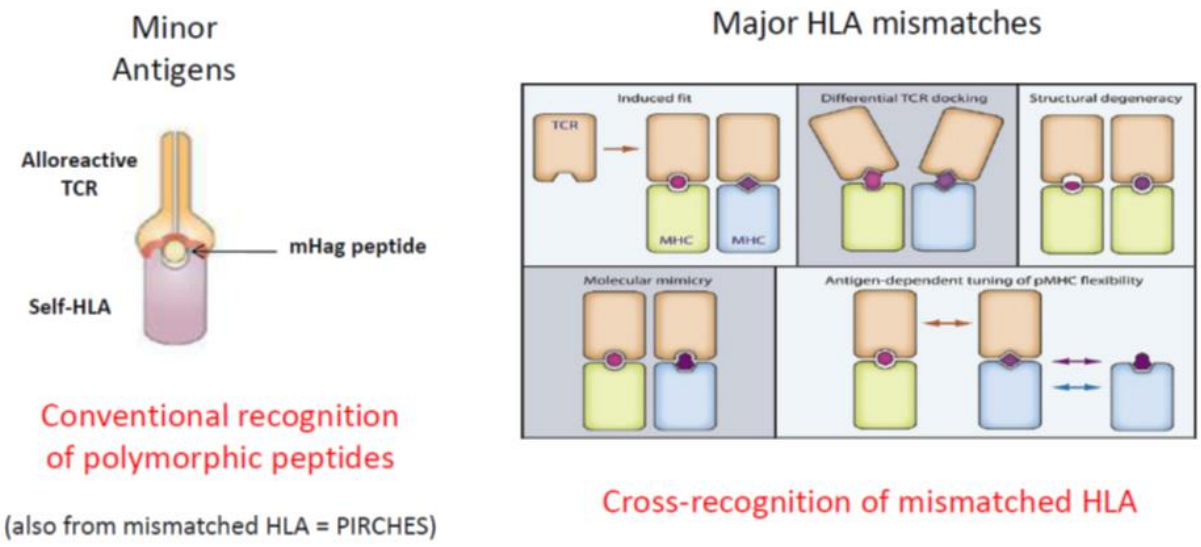


Figure 1.9: T cell alloreactivity to minor and major histocompatibility antigens.

miHA are recognized by self-HLA restricted TCR specific for a polymorphic non-self peptide encoded anywhere in the genome. When the non-self peptide is derived from mismatched HLA, this is called predicted indirectly recognized HLA epitopes (PIRCHE) (adapted from Geneugelijik [99] and Nathan [100]).

1.3.5 HLA nomenclature and tissue typing

Extensive molecular analysis of the HLA system has revealed that the extraordinary complexity at the genetic level of those closely linked genes is reflecting a very long and involved evolutionary history [82]. Advantageous variants are under strong positive selection and rise to high frequencies rapidly. A common uniform nomenclature was established by the HLA Nomenclature Committee in 1968 and is being constantly adapted as new HLA variants are being discovered [79]. The nomenclature for serological HLA antigens and for molecular HLA genes is different. Serological nomenclature describes HLA antigens and their so-called splits, i.e. subtypes recognized by a single broad serological antibody but different split antibodies, as defined by serological antibody testing (Serologic HLA typing). Serologic typing was the main technique for tissue typing until the 1990s, when molecular biology and PCR made their way into the diagnostic laboratories. Molecular HLA typing unraveled an unexpected degree of polymorphism, with up to over thousand different alleles able to code for the same serological antigen. Based on this, a molecular nomenclature was established, characterized by an asterix after the HLA locus designation and several fields behind it, indicating different levels of typing resolution (Figure 1.10).

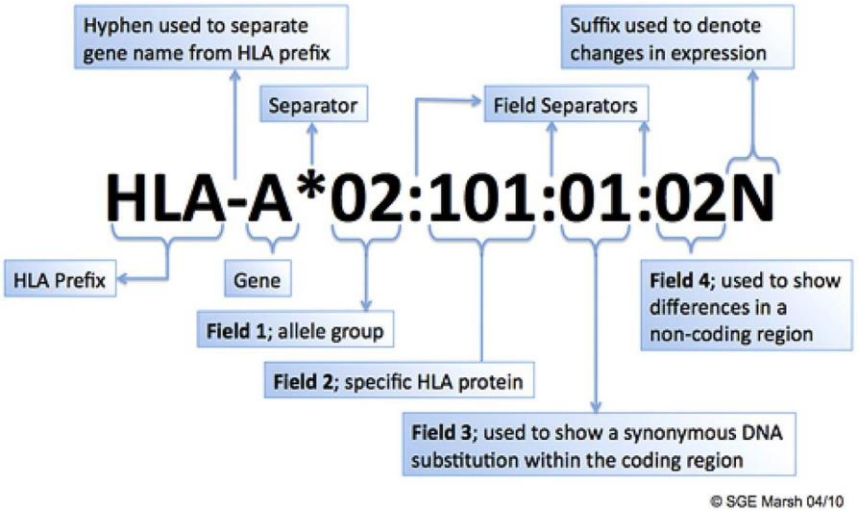


Figure 1.10: HLA Nomenclature.

HLA allele names have a unique number corresponding to up to four sets of digits separated by colons. The digits before the first colon describe the type, which often corresponds to the serological antigen followed by a set of digits used to list the subtypes (http://hla.alleles.org/nomenclature/naming.html) [101].

Molecular HLA typing in the 1990s was based on PCR amplification of the exons encoding the most polymorphic HvR, i.e. exons 2 and 3 of HLA class I and exon 2 of HLA class II. Polymorphisms were subsequently revealed by sequence specific priming (SSP), sequence specific oligonucleotide probing (SSOP) and/or Sanger sequence based typing (SBT). A major problem with these methods is ambiguous typings resulting from the co-amplification of two different alleles (one from the maternal and the other from the paternal haplotypes) in a single PCR reaction. This problem has been largely overcome in the last decade by the adaptation of NGS protocols to HLA typing, in which millions of sequencing reads (20 Mio. by MiSeq Illumina) are produced in a single experiment through clonal amplification [102,103].

NGS based HLA typing, introduced by now into the clinical routine of many laboratories, has revolutionized the field not only due to its ability to resolve ambiguities, but also to its high-throughput character. NGS typing has greatly increased our knowledge on the extent of HLA polymorphism, and the number of HLA alleles reported to the IMGT/HLA databases continues to increase (Figure 1.11). Most recently, NGS typing is being applied to full-length sequencing of the entire coding and non-coding region of HLA genes, unraveling further polymorphism and opening the stage to the targeted analysis of HLA mutations as cause of diseases, for instance in the field of tumor immunology.

Since more than two decades, harmonization of tissue typing protocols and quality assurance are safe-guarded by the European Federation for Immunogenetics (EFI; www.efi-web.org) and the American Society for Histocompatibility and Immunogenetics (ASHI; www.ashi-hla.org) through dedicated programs of laboratory accreditation.

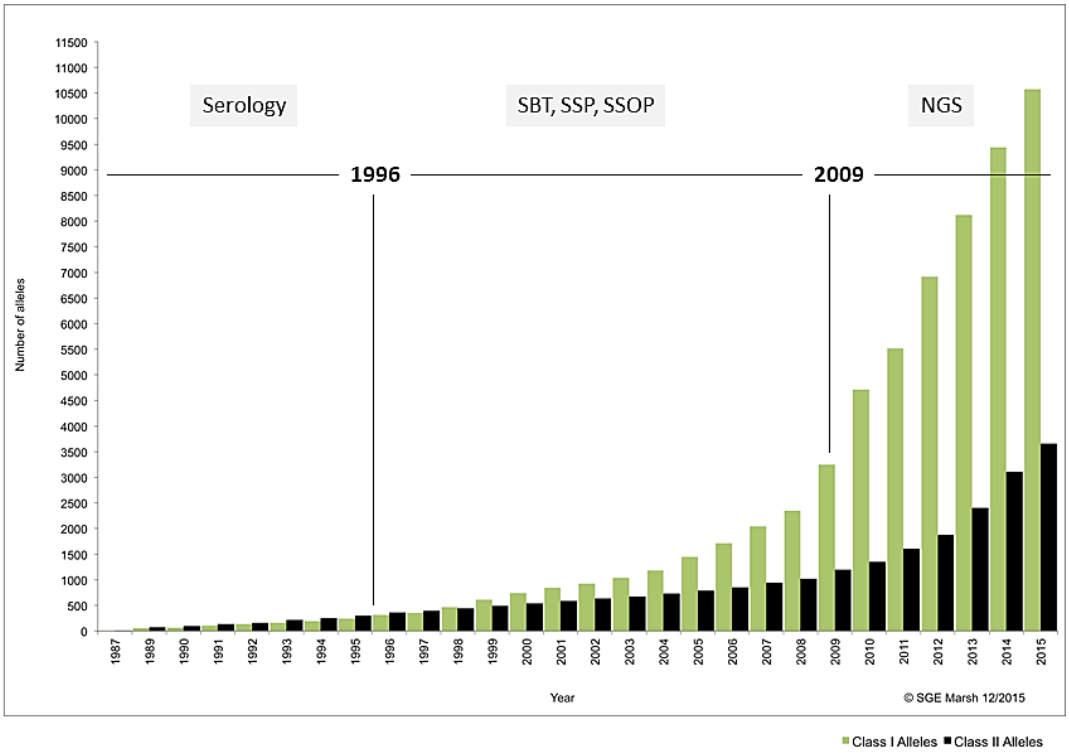


Figure 1.11: HLA allele numbers in the IPD-IMGT/HLA Database in relation to the development of tissue typing methodologies over time.

The figure shows the increasing number of alleles for HLA class I (green bars) and class II (black bars), with the HLA typing methodologies in use in the different time eras (from https://www.ebi.ac.uk/ipd/imgt/hla/ [86]).

1.3.6 HSCT donors by histocompatibility

The first description of the involvement of MHC genes in the rejection of allogeneic tumors was made by the British immunologist Peter Gorer in 1937 [104]. Some years later, the British scientist Peter Medawar showed that the rejection of allogeneic skin grafts was mediated by a specific immune response [105], an observation honored by the Nobel Prize in Physiology and Medicine in 1960. A few years later, Peter Gorer and the American geneticist George Snell described for the first time histocompatibility antigens (H-2) in mice responsible for allograft rejection [106].

Later Snell received the Nobel Prize in Physiology and Medicine for the discovery of histocompatibility antigens together with Jean Deausset and Baruj Benacerraf (see chapter 1.3.1). The work of Donnall Thomas in the early 1960s established that successful engraftment of allogeneic BM in dogs was possible in littermates but not in completely unrelated animals. This reflected genotypic identity for MHC antigens discovered a few years earlier. Consistently, the first patients to receive allogeneic BM were transplanted from genotypically HLA identical siblings, i.e. siblings who had inherited the same copies of chromosome 6 carrying the MHC from each parent. By Mendelian rules, the probability for two siblings to be genotypically HLA identical is 25%. In HLA-identical siblings, the only source of alloantigens are mHAgs encoded by polymorphic genes anywhere in the human genome (see paragraph 1.3.4). These alloantigens are the targets of alloreactive donor T cells causing GvHD after HSCT; however the same T cells mediate also GvL, reducing the risk of malignant disease relapse. In contrast, allogeneic targets and GvHD are absent in monozygotic twins matched for all mHAgs as well as for HLA antigens, however, the risk of disease relapse is also higher after twin transplantation (Figure 1.12) [107].

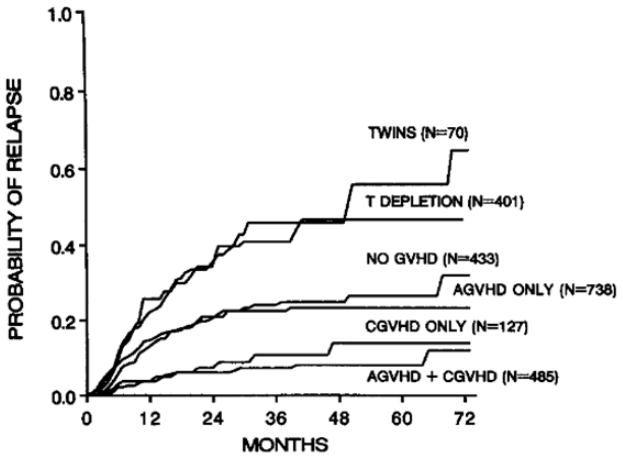


Figure 1.12: Kaplan Meier probability of leukemia relapse according to genetic disparity, T cell depletion and the development of GvHD.

The probability of leukemia relapse after allogeneic HSCT is higher for geno-identical twins compared to HLA-identical sibling donors. For the latter, the risk of relapse is increased by the use of T cell depletion, and by the absence of GvHD (from Horowitz [107]).

Therefore HLA-identical siblings remained the predominant graft source until the 1990s. However, only 25% of patients in need of HSCT have this type of donor available. When in 1979, the first successful BMT from an HLA-matched unrelated individual was performed at the Fred Hutchinson Cancer Center in Seattle [108] the first registries of volunteer donors were created during the 1980s both in the USA and in Europe, and to date count on over 30 million donors world-wide (www.bmdw.org). To date, HSCT from matched unrelated donors (MUD) have outnumbered those from HLA-identical sibling donors. Also in MUD, mHAg are generally present as targets of T cell alloreactivity leading to GvHD and GvL. However, far more frequent are mismatches at the HLA class II DP locus which is in low LD with DR and DQ (which instead are in strong LD between each other), and is also tolerated, since it gives rise to less vigorous T cell alloresponses than mismatches at HLA-DR. Also HLA-DQ mismatches are generally well tolerated. In contrast, it has been shown that the probability of survival after unrelated HSCT decreases by about 10% with every HLA mismatch at HLA-A,B,C or DR [109] (Figure 1.13).

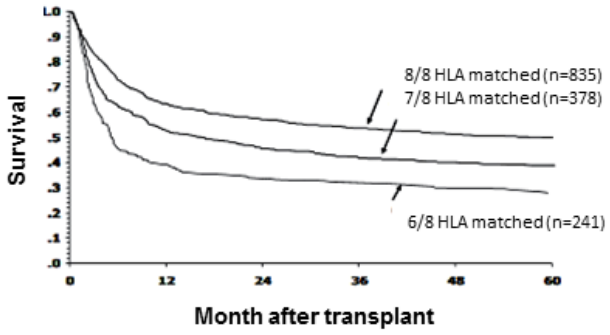


Figure 1.13: Impact of HLA matching on overall survival after unrelated HSCT.

Shown is the Kaplan Meier probability of survival for patients stratified according to the degree of HLA matching (8/8, 7/8, and 6/8) for HLA-A, -B, -C, and -DRB1(from Lee [109]).

According to the ethnic group of origin, the probability for a patient to find a MUD compatible for both alleles at the 4 loci HLA-A,B,C,DR (8/8 matched MUD), or at the 5 loci HLA-A,B,C,DR,DQ (10/10 matched MUD) varies greatly between 30 and 90% [110]. Therefore, MUD mismatched for 1 or more HLA alleles often have to be accepted (so-called mismatched MUD; MMUD). Over recent times, the use of MMUD has decreased in favor of family donors mismatched for an entire HLA haplotype (haploidentical donors). As discussed in paragraph 1.1.2, haploidentical HSCT became possible in the 1990s with the advent of G-CSF for the mobilization of

PBSC, which enabled the administration of mega-doses of HSC to overcome the histocompatibility barrier [12]. Initially however, these transplants were associated with high risks of infection and NRM, since they had to be performed in the presence of profound T cell depletion, leaving the patient immune system incompetent for a prolonged period of time. In 2006, a new type of GvHD prophylaxis based on the administration of cyclophosphamide in the early phases post-transplantation, pioneered at the John Hopkins University in Baltimore, paved the way for successful HSCT from haploidentical donors in the presence of T cells (T cell replete HSCT) [111]. Clinical results of this transplant modality appear similar to those obtained by matched MUD HSCT [112], and prospective randomized trials are under way to more definitely establish their relative efficacy. Haploidentical HSCT is an attractive option especially for low income countries due to the donor availability for nearly every patient and its relatively lower cost. Based on this, the clinical use of haploidentical HSCT continues to increase, and has outnumbered transplantation from UCB [13] (Figure 1.14).

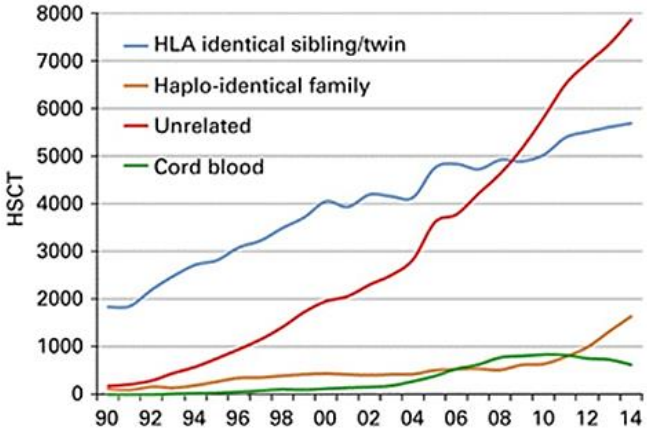


Figure 1.14: HSCT activity in Europe and adjacent countries (1990-2015).

Shown are the absolute number of sibling, haplo-identical, cord blood and unrelated first donor transplantations reported to the European Group of Blood and Marrow Transplantation (EBMT) over the years (from Passweg [13]).

The decrease in UCB HSCT reflects the limitations associated with HSC numbers, which often require the use of multiple UCB units for a given patient, and costs. Outcomes of UCB are however encouraging, with low rates not only of GvHD but also of relapse, suggesting that the superior content of naïve T cells in UCB grafts might mediate selective GvL, although the mechanism is still poorly understood.

This obvious advantage is however dampened by relatively high NRM rates reflecting delayed engraftment kinetics due to the generally low numbers of infused HSC. An overview of the different HSCT donor types, probabilities to identify them and frequent histocompatibility antigen mismatches is shown in Table 1.1.

Table 1.1: HSCT donor types, probability of identification and histocompatibility.

Donor Type	Probability %	Histocompatibility mismatches
Genetically identical Twin	NA	None
HLA-identical sibling	25	mHAg
MUD	30-90[$]	mHAg, HLA-DP[§]
MMUD	40-95[$]	mHAg, HLA-DP[§], 1-3 HLA alleles[#]
Haploidentical family	>95	mHAg, 1 HLA haplotype
UCB	30-70[$]	mHAg, HLA-DP[§], up to 4 HLA alleles[#]

[$]according to ethnic group of the patient; [§]in >80% of pairs; [#]HLA-A, B, C, DRB1, DQB1 alleles

1.4 Leukemia and other hematologic malignancies

1.4.1 Classification and characteristics

Hematologic malignancies arise through uncontrolled proliferation of cells from either the myeloid or the lymphoid blood lineage and affect patients of all ages, including young adults and children. Historically, hematologic malignancies located mainly in the blood or in the lymphatic system were referred to as leukemias and lymphomas, respectively. Based on the WHO Classification of 2001, the modern division is more related to the cell lineage derivation of the tumor [113]. Malignancies of the myeloid lineage include AML, MDS, and myeloproliferative disorders including chronic myeloid leukemia (CML), myelofibrosis and others. Neoplasms of the lymphoid lineage include ALL (which can be of B cell, T cell or NK cell lineage), Hodgkin and Non-Hodgkin lymphomas, CLL, plasma cell disorders (in particular multiple myeloma) and others. In children, ALL of B cell lineage is the most frequent type of blood cancer and has a relatively good prognosis by conventional treatment compared to adults (see paragraph 1.4.2). However, children can also be affected by other types of hematologic malignancies including AML which has a severe prognosis [114]. Generally, the incidence of blood cancers increases with age, whereby CLL represents the most frequent malignancy in the elderly, outnumbering any other type of solid tumors.

Due to their easy accessibility, hematologic malignancies are among the best studied. Nonetheless their biology is still only partly understood. They are frequently characterized by chromosomal translocations, which in part are pathognomonic for the disease, and which may or may not be causative drivers of tumorigenicity. Chromosomal rearrangements also provide an excellent tool for the targeted follow-up of treatment response by molecular tracing of the disease-specific marker (MRD). Monitoring of MRD can be based on different technological platforms including cytogenetics, fluorescence in situ hybridization (FISH), endpoint or nested PCR, qPCR, ddPCR, and NGS. In certain cases, specific drugs counteracting the function of molecules expressed in association with the chromosomal rearrangement have been instrumental for the design of new and very efficient targeted therapies (see paragraph 1.4.2.3).

1.4.2 Treatment

The prognosis of hematological malignancies varies greatly with disease type and patient characteristics. For instance, non-aggressive CLL arising from a mature B cell clone characterized by the presence of immunoglobulin hypermutation has an excellent prognosis without any type of interventional treatment (“wait and watch”) and often does not limit the life expectancy of the generally old patients it affects. In contrast, other hematologic malignancies such as certain types of acute leukemias have a dismal prognosis and can be cured only by immune intervention through allo-HSCT or targeted therapies.

1.4.2.1 Conventional treatment

Conventional treatment consists in high dose chemotherapy and, in the case of certain lymphomas, radiotherapy of affected sites. Chemotherapy can be combined with monoclonal antibodies (mAb) against molecules expressed by the tumor, for instance the CD20-specific mAb Rituximab for the treatment of B cell lymphomas. Chemotherapy is divided into induction and consolidation treatment. The aim is to achieve complete remission (CR1) after induction therapy. If relapse occurs and a second remission is induced (CR2), generally the prognosis worsens. It has also been shown that the biological and molecular features of the disease may change through chemotherapy, with relapse often resistant to the initial drugs used (see paragraph 1.4.2) [115].

1.4.2.2 HSCT

A definitive cure by conventional treatment can be achieved only for a fraction of hematologic malignancies. The risk of relapse after conventional treatment is dependent both on biological features of the disease, with certain cytogenetic and molecular rearrangements defining high-risk disease profiles, and clinical characteristics such as the disease status at diagnosis. A potentially curative option can be offered to these patients by HSCT. Auto-HSCT is mainly an option for diseases not homing to the BM, since BM-derived HSC from the patient are re-infused in this procedure. Auto-HSCT is therefore applied to diseases such as lymphoma or multiple myeloma, in order to enable the administration of MA high-dose chemotherapy. For hematologic malignancies homing to the BM, and with a severe prognosis as defined above, allo-HSCT often represents the only possibility of definitive cure. This is not only due to the possibility of increasing the intensity of chemotherapy regimens as described for auto-HSCT, but foremost and importantly to the effect of immunotherapy mediated by the healthy donor immune cells versus residual tumor. For instance, in the case of high-risk AML in CR1, a significant advantage in long-term survival has been demonstrated in a matched-pair analysis (Figure 1.15) [116].

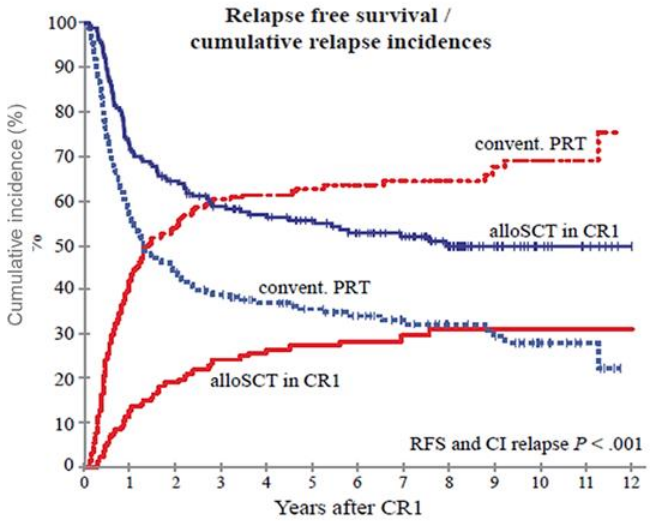


Figure 1.15: Survival and relapse after conventional treatment or allo-HSCT for AML in CR1.

Matched pair analysis of 200 patients in each group treated by conventional post-remission therapy (PRT) or allo-HSCT (alloSCT) for relapse-free survival (blue curves) or relapse incidence (red curves) (from Stelljes [116]).

However, as discussed in paragraph 1.3.6, the target molecules of GvL are often also expressed on healthy tissues of the patient, and GvHD accompanies GvL in many cases. GvHD and infectious events related to post-transplant immune suppression determine the toxicity of allo-HSCT which is directly associated with NRM. Moreover, despite allo-HSCT, disease relapse occurs in approximately one third of the patients and represents the major cause of death after day 100 post-transplantation [16,117] (Figure 1.4). Although survival is better when relapse occurs late after HSCT, the prognosis is overall dismal, especially in the first two years after transplantation (Figure 1.16).

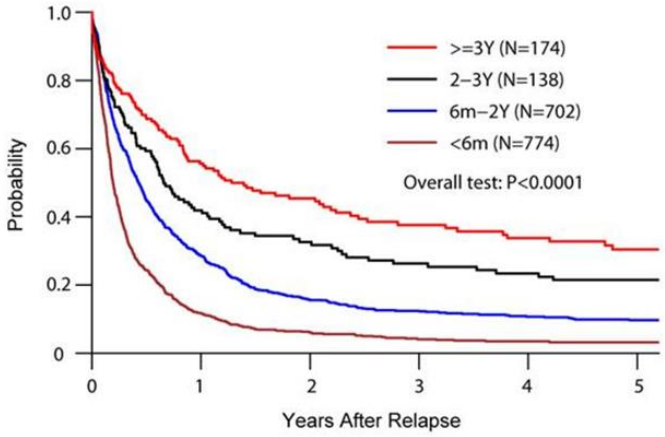


Figure 1.16:Survival by time from allo-HSCT to AML relapse.

Evaluated survival of AML patients relapsing after alloHCT of 1788 pediatric and adult AML CR1 & CR2 patients relapsing post-transplant (1990-2010) after a median follow up of 39 month (range, <1-193). 229 patients (13%) survived 5-years, 3% (95% CI 2-5%) for patients relapsing within 6month, 11% (95% CI 8-14%; p<0.0001 comparing to <6 month) for 6month-2years, 21% (95% CI 14-28%; p<0.0001 comparing to <6 month) for 2-3years and 31% (95% CI 23-39%; p<0.0001 comparing to <6 mo) for ≥3years (from Bejanyan [118]).

Treatment of relapse should commence as early as possible, i.e. ideally when it is still at a molecular or cytogenetic state. For this reason, the availability of sensitive methods to detect imminent relapse is of paramount importance. These include tracing of MRD markers as well as, in particular if no disease specific markers are available, patient-specific chimerism markers as discussed in paragraph 1.4.2. The first treatment modality of molecular or cytogenetic consists in down tapering of immune suppression, accompanied by DLI if possible, to increase GvL. However, this exposes the patient also to increased risks of GvHD. To reduce this risk, DLI genetically modified to express suicide genes, which can be pharmacologically activated after infusion in case of need, have been pioneered since 1990 [117,119,120].

In 2016, T cells engineered with the thymidine kinase (TK) suicide gene were the first to receive approval by the European authorities as a pharmacological immunogene therapy under the name Zalmoxis®. Other suicide platforms including the use of inducible Caspase 9 [35] are also entering the clinics. In case of overt hematological relapse characterized by the presence of more than 5% blasts in the BM and/or PB, treatment consists in re-induction chemotherapy and possibly a second HSCT, if the patient is fit for this option. In 2015, re-transplantation amounted to 1272 (7.4%) of the total 17,302 allo-HSCT reported to the EBMT [13]. Targeted drugs including mAb against tumor-specific antigens and tyrosine kinase inhibitors (TKI) if applicable are also an option, as well as hypomethylating agents such as azacytidine (Aza) which might act by changing the transcriptional landscape through epigenetic modifications [121,122]. Although certain features such as diagnosis and disease status at transplant are associated with the risk of relapse after allo-HSCT, many of the biological causes for its occurrence remain obscure, and studies aimed at shedding light onto this question are urgently warranted to design new strategies for its prevention and cure.

1.4.2.3 Novel treatment modalities

Despite the superior efficacy of allo-HSCT compared to conventional treatment to cure high-risk hematologic malignancies, toxicity and mortality associated with infection and GvHD remains high, fueling interest in the search for alternative treatment modalities. TKI were among the first targeted therapies to make their way into the clinical practice. In particular, TKI imatinib (Gleevec) was successfully introduced in the beginning of the 2000s to treat CML and other Philadelphia chromosome-positive malignancies with constitutive activation of the Abelson murine leukemia viral oncogene homolog 1 (abl) tyrosine kinase through fusion with the break point cluster region bcr [123]. This revolutionized indications for allo-HSCT, which had been dominated by CML in the 1990s, whereas today CML is transplanted mainly in case of the onset of TKI resistance after treatment with the drug (i.e. imatinib) and represents only 2% of the overall indications for allo-HSCT [124] (Figure 1.2). Similarly, a reduction in the transplant rates for CLL was reported in 2015, possibly reflecting new kinase inhibitors and bcl2 inhibitors developed to treat CLL [13]. In the last few years new modalities of specific targeted immunotherapy have been developed by the use of chimeric antigen receptor (CAR) T cells and bi-specific antibodies.

These have the potential of providing selective tumor surveillance without the toxic side effects of allo-HSCT. Both rely on the use of the antigen recognition site of a mAb recognizing a tumor cell surface molecule with CD19 for B cell malignancies being one of the best explored target molecules. For CAR, the antigen recognition site is linked to a co-stimulatory molecule and the signaling domain of the T cell receptor, leading to potent activation of T cells transduced with the CAR upon encounter of the antigen on tumor cells. Bispecific antibodies promote T cell activation through crosslinking of CD3 on T cells via their second antigen binding domain. Both CAR and bi-specific antibody therapy have entered the clinics for the treatment of Hodgkin lymphoma and other CD19+ B cell malignancies with very promising results [125]. The initial enthusiasm was however dampened by the relatively short duration of clinical response rates, which are often limited to months. The complex reasons for this include problems related to the persistence of transduced CAR T cells, as well as tumor immune escape variants through down-regulation of the CD19 target molecule. Moreover, also CAR T cell therapy is not without toxicity, as it can be associated with a severe and even potentially life-threatening condition called cytokine release syndrome (CRS). CRS occurs within days after treatment and is caused by overactivation of the infused cellular product. Another problem is the time and costs related to the personalized production of an autologous CAR product for every patient. Endeavors to generate universal CAR T cells from third party donors are under way. However, efficient means will have to be found to prevent their elimination by the patient immune system. CAR T cell and bispecific antibody therapy holds promise both, as bridge to transplantation and as potential treatment of imminent or overt post-transplant relapse.

An additional new treatment modality of great promise is the use of so-called checkpoint inhibitors (CPI), i.e. mAb directed against inhibitory molecules on T cells or their respective receptors on tumor cells, including ipilimumab (anti-CTLA-4), nivolumab (anti-PD1) and atezolizumab (anti-PDL1). These antibodies unleash the patient's T cell response and have the advantage of not targeting a defined antigen on the tumor. But they activate polyclonal responses by reducing the risk of immune escape. While CPI are being successfully applied for the treatment of different solid tumors including melanoma and lung cancer, their use in hematologic malignancies is only at the beginning. They are also being considered for treatment of relapse after allo-HSCT, which however is associated with an increased risk of concomitant GvHD.

1.4.3 Clonal evolution and immune escape

Leukemia has been shown to have a hierarchical architecture, with the most immature leukemic stem cells (LSC) at the apex fueling proliferation of the progeny in the tumor mass [126]. Durable cure thus depends on the capacity of a given treatment to eliminate all LSC, which may be composed of different subclones. LSC evade the host protection process by acquisition of somatic mutations and maintain the key stem cell properties of self-renewal, extensive proliferative and differentiative potential [127-129]. AML is not amongst the tumors with the highest mutation rates [130], but comparison analysis of samples at diagnosis and at relapse showed complex changes in karyotypes, gene aberrations, downregulation of co-stimulatory or HLA molecules [127 131]. Genomic instability and selection of pre-existing subclones is likely one aspect of the evolution of therapy resistance. However, the understanding of the biological processes generating these mutations is limited.

The hypothesis that accumulation of >2 mutations is needed for cancer formation was first proposed by Knudson in 1971 [132]. Genes involved in the pathogenesis of cancer act by two general mechanisms: the structural alteration of a normal gene (proto-oncogene) to generate a novel gene (oncogene) and the loss or inactivation of onco-suppressor genes involved in the regulation of cell cycle or apoptosis [133]. The first mechanism of leukemia transformation relates to protein products which are usually involved in cellular proliferation, differentiation, or survival and the second to evade growth control and tumor suppression. This explains certain genetic and chromosomal abnormalities involving oncogenes such as p53 N-ras DEK/CAN bcl-2 and PML [133] ,which have been correlated with specific types of leukemia.

Clonal evolution of leukemia relates to the concept of Darwinian Theory of evolution; natural selection and survival of the fittest, means that leukemic cells with heritable traits better suited to the environment of the transplanted immune system will survive in an immunosuppressive environment. Shankaran and Dunn [134,135] showed by the use of in vivo models that the immune system is not only protective against tumor formation but also selects specific tumor variants with reduced immunogenicity. These findings led to the idea of three essential phases of cancer immune editing: Elimination, Equilibrium and Escape [134].

Elimination: This is the first step needed for eradication of developing tumor cells, which includes the innate (by inflammatory cytokines, activated effector cells and amplification by more pro-inflammatory cytokines) and the adaptive immune response (NK cells that promote maturation of DCs which in turn are driving clonal expansion of CTLs). ***Equilibrium:*** Cells that survive elimination enter a phase of equilibrium. In this phase the interaction with the adaptive immune system sculpts the immunogenicity of the tumor cell. ***Escape:*** Cells acquire the ability to evade immune recognition by many different mechanisms such as HLA class I alterations and establishment of an immunosuppressive microenvironment.

Since there is constant immune pressure that forces residual leukemia cells to undergo clonal progression, AML relapse and resistance after chemo and/or HSCT are caused by genetic and epigenetic events that are frequently derived from subclones not present at diagnosis (Figure 1.17) [136]. Li Ding and colleagues compared AML at diagnosis and at relapse after chemotherapy and found that while diagnosis was composed of several subclones characterized by different somatic mutations recurrent in AML and relevant for pathogenesis, a single subclone emerged after chemotherapy, which had acquired additional somatic mutations which probably provided a selective advantage [136].

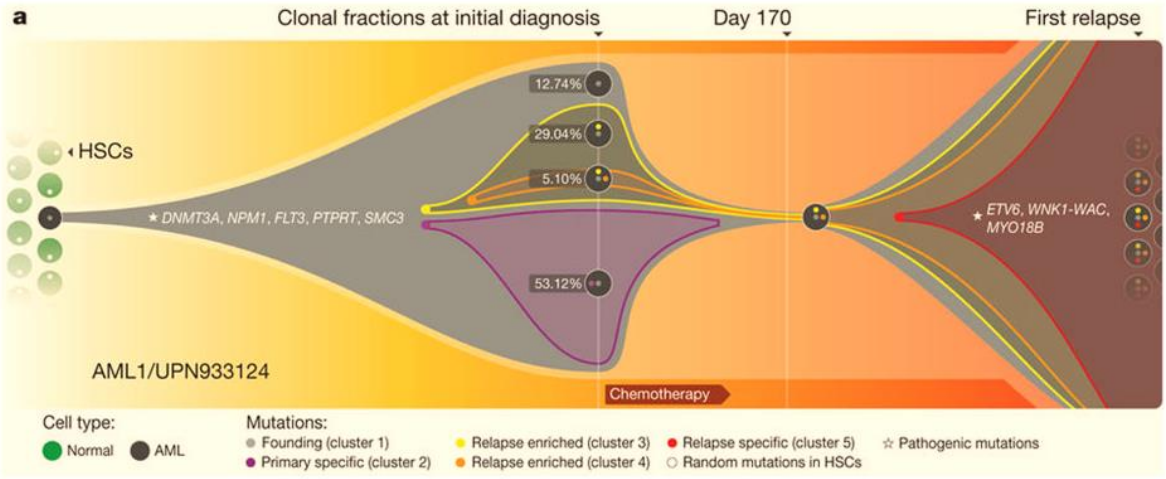


Figure 1.17: Clonal evolution in relapsed AML.

Graphical representation of clonal evolution from the primary tumor to relapse in a representative patient and patterns of tumor evolution observed in the primary tumor and at relapse (from Li Ding [136]).

Immune escape is a special form of clonal evolution, whereby the tumor cell acquires specific alterations that interfere with immune surveillance, and play a special role in relapses after tumor immunotherapy, which puts the neoplastic cells under specific immunological pressure. However, immune escape mechanisms have also been described in patients not treated by immunotherapy, protecting the tumor from elimination by the patient's autologous immune system. It is well established that many tumors establish a tolerogenic environment, for instance by the secretion of certain immune suppressive cytokines or by up-regulation of specific ligands interacting with inhibitory receptors on immune cells (the latter is at the basis of the success of CPI in treating certain types of tumors, see paragraph 1.4.2.3). A central molecule for antigen presentation and recognition is HLA, and not surprisingly have genomic or phenotypic alterations of the MHC and antigen-presenting machinery been frequently observed in solid tumors [137-140]. These alterations include loss of single HLA alleles as well as haplotype loss (loss of heterozygosity; LOH). LOH frequently oncurs by deletion of one haplotype and subsequent duplication of the other, a process called uniparental disomy (UPD) [141,142]. Total HLA loss by tumor cells has also been described, however this event is rare since HLA class I negative cells are generally eliminated by NK cells due to lack of inhibition by inhibitory receptors specific for HLA class I ligands on these cells [143]. Recently, somatic neomutations in HLA class I genes have been described as an additional mechanism for loss of function alterations in solid tumors [140].

In the context of allo-HSCT, the first description of selective loss of mismatched HLA by relapsing leukemia was reported by Grosse-Wilde and colleagues, who showed as early as in 1987 that a patient relapsed with specific loss of mismatched HLA six years after BMT [144]. This finding was more recently confirmed by Vago and colleagues who demonstrated selective genomic loss of the mismatched HLA haplotype ("HLA loss") by AML relapsing after haploidentical HSCT [142]. The underlying mechanism was UPD encompassing the entire short arm of chromosome 6, including the MHC leading to the evasion from donor T cell immune control and clinical relapse (Figure 1.18) [142,145].

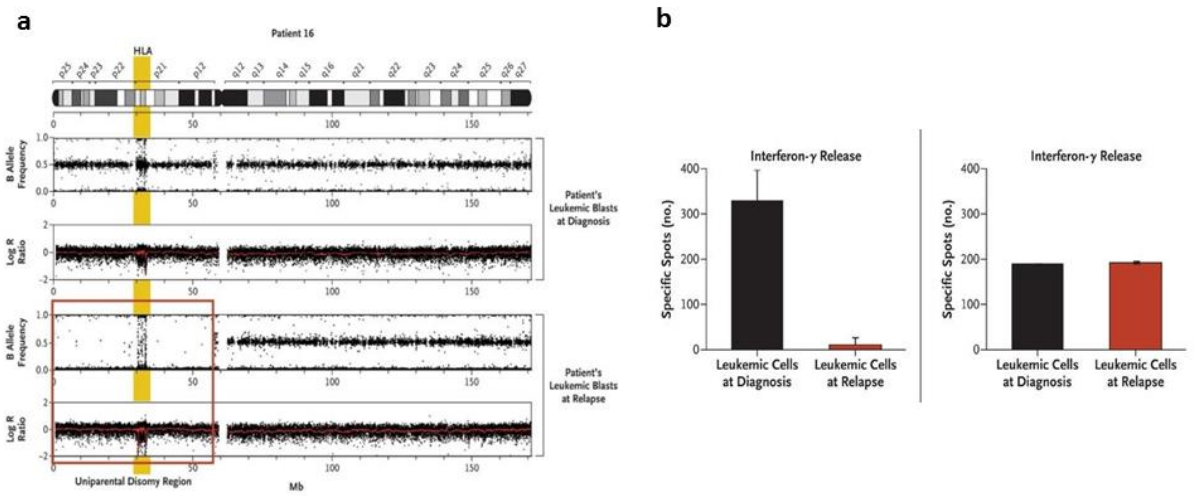


Figure 1.18: Selective loss of mismatched HLA by AML relapsing after haploidentical HSCT.

(a) Comparative SNP array analysis of leukemia at diagnosis and relapse showed UPD encompassing the entire short arm of chromosome 6 including the MHC (in yellow), in the presence of normal copy number variation (CNV). (b) Selective HLA loss abrogates recognition by alloreactive donor T cells, which continue to recognize leukemia at diagnosis. Leukemia at relapse is recognized by third party T cells alloreactive against the residual HLA haplotype, thereby demonstrating specificity of the evasion (from Vago [142]).

Since then HLA loss relapses were described after haploidentical transplantation by different groups [146-149], and in different clinical platforms used for haploidentical HSCT including GvHD prophylaxis by PT-Cy [147]. The emerging picture is that this type of immune escape is at the basis of one third of leukemia relapses after haploidentical HSCT [150,151]. Interestingly, risk factors for HLA loss relapse were related to immune pressure by transplantation, namely the presence of GvHD prior to relapse and the number of T cells infused. Moreover, the median time to relapse was significantly longer for HLA loss compared to “classical” relapses (94 vs 300 days post-transplant, respectively), probably reflecting the time required for clonal evolution and outgrowth of the HLA loss variant.

The incidence of HLA loss relapse after HSCT from HLA mismatched donors other than haploidentical family members is less well understood. Sporadic reports of its occurrence in the 9/10 MMUD setting as well as after HLA-identical sibling transplantation have been published [150,151], but its overall incidence and risk factors in these settings, as well as after 10/10 matched MUD and UCB transplantation, is unknown.

Similar to “classical” relapses, the outcome of HLA loss relapses is poor. Importantly, the efficacy of the standard relapse treatments, i.e. down-tapering of immune suppression and DLI, is severely hampered by the very presence of HLA loss, since the mismatched HLA targets of T cell alloreactivity are eliminated (Figure 1.18). This strongly shifts the risk-benefit ratio of DLI versus toxic GvHD, since the HLA mismatches are preserved on healthy tissues. Knowledge about the presence of HLA loss relapse is therefore not purely academic, but has direct and important implications for clinical decision making. If possible, the patient should be offered a second transplant from an alternative haploidentical donor mismatched for the haplotype that is preserved by the HLA loss leukemia (Figure 1.19). Interestingly, it was shown that patients, who were treated by a second transplant for leukemia relapse after haploidentical HSCT had a significant survival advantage when the donor of the second transplant was mismatched for the other haplotype with respect to the first (Figure 1.19) [152]. In addition to a second transplant from an alternative haploidentical donor, treatment options for these patients should be explored amongst the new therapies, such as the infusion of non-HLA restricted donor immune cells such as NK cells or γδ Tcells, or the application of CAR T cells or bi-specific antibodies (see paragraph 1.4.2.3).

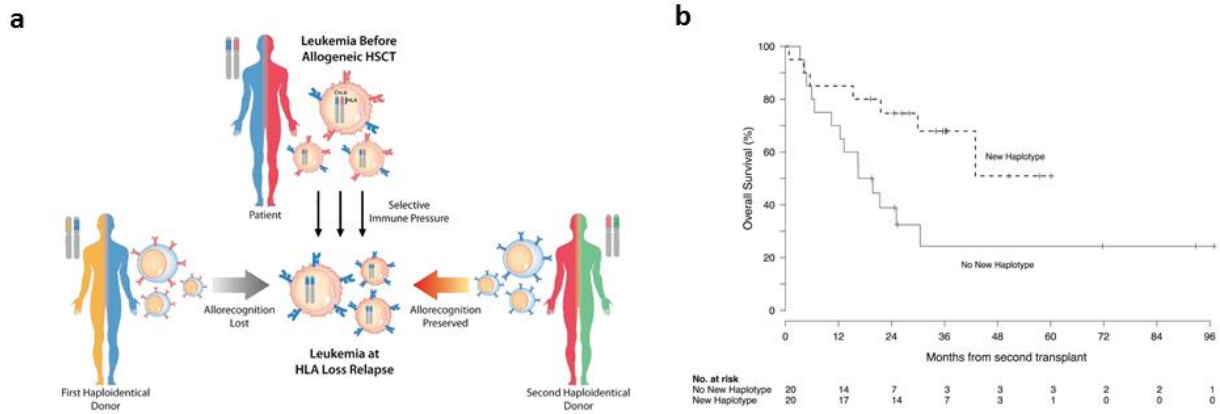


Figure 1.19: Treatment of HLA loss relapse by second HSCT from an alternative haploidentical donor.

(a) Rationale for the efficacy of HLA loss relapse treatment by a second HSCT from a donor haploidentical to the patient’s other haplotype (in red; second haploidentical donor), compared to a donor haploidentical to the same haplotype as before (in blue; first haploidentical donor) (from Vago [153]). (b) Probability of overall survival after treatment of post-transplant relapse with a second HSCT from a donor haploidentical for the other (“New haplotype”) or the same (“No new haplotype”) haplotype (from Imus [152]).

Based on these considerations, routine evaluation of the presence or absence of HLA loss relapse, and possibly its early detection in a status of molecular rather than overt hematological relapse, is clinically desirable. However, it is difficult to implement such methodologies for most centers because of methodological and interpretative hurdles. Detection of this condition has to be based on comparative quantification of patient-specific genomic polymorphisms outside and inside the HLA system. This is currently done by separate techniques with different sensitivities, such as STR for polymorphisms outside HLA (conventional chimerism analysis, see paragraph 1.4.2), and SSOP or genome-wide SNP arrays for polymorphisms inside the HLA (HLA typing methods, see paragraph 1.4.3). The sensitivity of these methods is limited to 1-5%, and reliable information on the presence or absence of HLA loss can only be obtained from purified leukemic blasts, which in turn can be sorted only in the presence of overt hematologic relapse. Moreover, these cumbersome techniques are not amenable to routine diagnostics, reserving the detection of HLA loss to retrospective analysis in highly specialized research laboratories. The development of sensitive and reliable methods for the detection of HLA loss in a single, user-friendly assay suitable for application in the clinical routine represents therefore an unmet medical need. Moreover, high-throughput analysis of HLA loss by NGS-based methods is an equally relevant pre-requisite to the establishment of international collaborative efforts aimed at elucidating the incidence and risk factors of this condition in transplant settings other than haploidentical HSCT, including MUD, MMUD and UCB.

2. Aim

Leukemia relapse is the most frequent cause of treatment failure of allo-HSCT. After HSCT from haploidentical donors, it is frequently caused by immune escape through selective genomic loss of the patient-specific mismatched HLA (HLA loss relapse). The timely diagnosis of this condition is relevant for adopting appropriate treatment strategies. However, accurate, sensitive and user-friendly assays amenable to the clinical routine are yet not available for this purpose. Moreover, high-throughput methods for its characterization in different and widely used transplant settings, including unrelated donor and cord blood transplants, are lacking so far.

The aim of the present Ph.D. project was to close these gaps by:

1) Investigating the utility of qPCR as an innovative tool for chimerism monitoring after HSCT (*Ahci et al. Biol Blood Marrow Transplant 2017*);

2) Within that tool developing HLA allele-specific qPCR assays for the diagnosis of HLA loss relapse (*Ahci et al., Blood 2017*);

3) Establishing new next generation sequencing based protocols for the high-throughput detection of HLA loss relapses.

3. Materials and Methods

3.1 Investigation of qPCR as a tool for chimerism monitoring

3.1.1 Patients and transplants

Thirty adult patients who received a first allo-HSCT mainly from unrelated donors for AML, ALL, or other malignancies between 2006 and 2013 at the University Hospital Essen were included in the analysis of qPCR for chimerism monitoring. Enrollment criteria included diagnosis, donor type, and the availability of several follow-up samples and their STR chimerism results. Patient, donor and transplant characteristics are shown in Table 4.1 (see page 49). All but 2 patients received MA conditioning, followed by infusion of unmanipulated donor PB stem cells and GvHD prophylaxis based on short course methotrexate and cyclosporine A for at least 210 days. Immune prophylaxis included ATG (Fresenius; Neovii Biotech, Gräfeling, Germany) at a total dose of 60 mg/kg pre-HSCT in 19 patients. Transplants were performed after written informed consent, under clinical protocols approved by the Ethical Review Board of the University Hospital Essen, in accordance with the Declaration of Helsinki.

3.1.2 Clinical outcome endpoints

An overview of the clinical outcome of the thirty patients enrolled in this study is given in Table 4.2 (see page 50). The two primary clinical outcome endpoints were engraftment and disease relapse. Follow-up was recorded until January 29, 2015, with a median time of follow-up of 1504 days (range, 317 to 2981). Time to engraftment was defined as days post-HSCT needed for achievement of at least 500 white blood cells per µL. Patients were considered informative for engraftment when follow-up samples were present from different time points in the first 210 days post-HSCT and did not present with disease relapse during that time. Engraftment kinetics were classified as normal (i.e., sustained <0.1% patient chimerism on consecutive PB samples), delayed (i.e., sustained 0.1% to 1% patient chimerism on consecutive PB samples that eventually dropped below 0.1%), or persistent mixed chimerism (PMC) (i.e., sustained >1% chimerism on consecutive PB samples).

Hematologic and cytogenetic disease relapse was defined as at least 5% morphologic blast counts and the reappearance of previously detected clonal cytogenetic abnormalities, respectively. Patient samples were considered informative for relapse when at least 1 sample was present at most 180 days before relapse onset in patients who had achieved full donor engraftment before that time.

3.1.3 Post-transplant follow-up samples

All patient PB and BM aspirate samples were harvested in concomitance to routine diagnostic procedures and upon written informed consent approved by the University Hospital Essen Ethics Committee and stored at -20°C for further analyses. At least 5 longitudinal PB or BM follow-up samples, drawn at different time points between day 21 and day 2302 post-HSCT, were available for all patients, for a total of 459 samples (364 PB and 95 BM). The number of samples and time spans of sample collection post-HSCT are shown for each patient in Table 4.2. Genomic DNA was extracted automatically from PB or BM using MagNa Pure LC 2.0 (Roche Diagnostics, Indianapolis, IN) according to the manufacture's protocol. Concentration and purity was quantified by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

3.1.4 Chimerism testing by STR

All follow-up samples were routinely analyzed at the time of collection for hematopoietic chimerism (HC) by STR at the Department of Bone Marrow Transplantation of the University Hospital Essen, Germany. STR HC was performed by the MentypeChimera PCR Amplification Kit (Biotype Diagnostic GmbH, Dresden, Germany), which is based on 12 polymorphic autosomal STR loci, according to the manufacturer's instructions. Results were analyzed for using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The input DNA was 1 ng, resulting in a positivity threshold of 1% as described [58 56 60 57 59].

3.1.5 Chimerism testing by qPCR

All follow-up samples were retrospectively analyzed for HC by qPCR at the Institute for Experimental Cellular Therapy of the University Hospital Essen, Germany. qPCR HC was performed using the AlleleSEQR chimerism kit (Abbott Molecular, Wiesbaden, Germany) according to the manufacturer's recommendations. The kit covers 34 bi-allelic insertion-deletions markers spread over 20 different chromosomes.

After screening, two patient-specific markers (positive in the patient but negative in the donor) were selected and analyzed in parallel, each in duplicate with <0.5 standard deviation. HC percentages were determined using the delta-delta cycle threshold method comparing cycle threshold values of the insertion deletion in reference to those of an internal housekeeping gene, between the sample under analysis and the patient pre-transplant, as described [154]. The final HC value was determined as mean values between the 2 separate assays (preferentially located on 2 different chromosomes). The input DNA for each reaction was 100 ng, resulting in a positivity threshold of 0.1% as described [57 63].

3.1.6 Detection of CMV reactivation

Post-transplant CMV reactivation in the first 100 days post-HSCT was monitored by in-house immunofluorescence staining for the pp65 antigen as described [155] in 22 patients by a commercial qPCR kit for the CMV genome (Abbott Realtime CMV; Abbott Molecular) and for the remaining 8 patients, according to the manufacturer's instructions. Thresholds for CMV reactivation were ≥25 positive cells per million PB cells for pp65 and ≥1000 copies for qPCR, as previously published [28 63].

3.2 Development of HLA allele specific qPCR assays for the diagnosis of HLA loss relapses

3.2.1 Primer design

In this study, qPCR reactions were developed for frequent alleles of the loci HLA-A, -C and -DPB1 as listed in Table 4.5 (see page 62) and Table 4.6 (see page 63). Nucleotide sequences of the alleles reported for each of these loci were downloaded from the IMGT database (Release 3.27.0, 2017-01-20; http://www.ebi.ac.uk/ipd/imgt/hla/). Allele groups were defined and primer design was carried out using BioEdit biological sequence alignment editor version 7.2.5 (http://bioedit.software.informer.com). Primers discriminating specific SNPs in the exon or intron of the selected HLA allele group at their 3' end were evaluated using the NCBI-Nucleotide Blast (https://blast.ncbi.nlm.nih.gov/Blast) and Oligo Analyzer version 3.1 (https://eu.idtdna.com/calc/analyzer) softwares.

3.2.2 Genomic DNA Extraction

Genomic DNA was extracted from 2-5x10^6 cells (PBMC or BLCL) using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Concentration and purity was quantified by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Purified DNA should have an A260/A280 and A260/A230 absorbance ratio between 1.7 and 1.9.

3.2.3 Technical validation of qPCR assays for HLA markers

Primers were first tested by conventional end-point PCR on positive and negative reference DNA and the amplicon sequence was verified by Sanger Sequencing. Primers were then tested in qPCR with SYBR green (Applied Biosystems, Life Technologies, California, USA) and finally with Taqman chemistry conditions using minor groove binding (MGB) probes, which are designed with 2 fluorophores: FAM and Quasar-670 (similar to Cy5). All qPCR reactions were carried out in technical duplicates on a 7500 Real-Time PCR System using the same qPCR conditions (reference gene, buffer, enzyme, thermal cycler settings) as for the commercial KMRtrack assays (GenDx, Utrecht, Netherlands). Data analysis was performed using the 7500 Software v2.3 (Applied Biosystems, Life Technologies, California, USA).

Specificity testing was performed for each assay using 300ng input DNA from the panel of positive and negative reference DNA. Assays with multiple specificities were tested independently for each of the relevant HLA alleles or allele groups.

Efficiency and sensitivity testing was performed for each assay using serial 5-fold dilutions in water or in negative reference DNA, respectively, starting from 100ng of input positive reference DNA per qPCR reaction with an end volume of 20 µL (according to commercial KMRtrack conditions). Amplification efficiency (E) of the qPCR reactions is the optimal value (here E=90-105%; meaning that an uninhibited PCR reaches a doubling of template DNA per cycle) calculated from the slope of a serial dilution via the formula:

$E(\%) = 10^{(-1/slope)}$, in which the slope characterizes the linearity of the standard curve.

Maximal reproducible sensitivity was defined according to MIQE guidelines as the last dilution with a ΔC_T of ≥3 between target positive and negative DNA [156]. "Artificial chimerism" was determined by assessing the percentage of chimerism on an artificial curve, positive reference DNA serially diluted into negative reference DNA, relative to undiluted positive reference DNA, according to the $\Delta\Delta C_T$ formula:

$2^{-\Delta\Delta CT}$ x 100%, in which $\Delta\Delta C_T$ equals to:

$(C_T\ (marker\ chimeric) - C_T\ (reference\ chimeric)) - (C_T\ (marker\ undiluted\ positive) - C_T\ (reference\ undiluted\ positive))$.

3.2.4 qPCR assays for non-HLA markers

Non-HLA marker screening and chimerism analysis were performed using the commercial KMRtype and KMRtrack assays (GenDx, Utrecht, Netherlands), according to the manufacturer's recommendations. The kit covers 39 bi-allelic markers spread over 20 different chromosomes. Briefly, patient-specific markers (positive in the patient but negative in the donor) were selected by KMRtype, using 10ng of input DNA of patient or donor in 10 multiplexed assays. Chimerism quantification was performed in triplicate using 100ng of input DNA of the test sample post-transplant. The percentage of chimerism was calculated using the patient pre-transplant DNA as reference, according to the $\Delta\Delta C_T$ formula, as described above.

3.2.5 HLA-typed reference DNA

A panel of 45 different HLA-typed reference DNAs was obtained from B lymphoblastoid cell lines (BLCL) or peripheral blood mononuclear cells (PBMC) of healthy blood donors. BLCL were purchased from the European Collection of Authenticated Cell Culture, or established from PBMC samples of healthy donors by standard procedures [157]. PBMC were obtained from buffy coats of healthy blood donors referring to the University Hospital Essen, under informed consent and specific approval by the local ethical committee. HLA typing was performed by SSO and SSP at the Institute for Transfusion Medicine of the University Hospital Essen or Ospedale San Raffaele (OSR) in Milan, Italy according to EFI quality standards. The panel covered the most frequent relevant HLA types in worldwide populations, as listed in Supplementary Table 9.1 (see page 108).

3.2.6 Patient samples

The PB and BM aspirate samples used for the validation of the developed qPCR assays for HLA markers were obtained from patients who experienced HLA loss or classical relapse after haploidentical HSCT performed at the center of our collaborators at the San Raffaele Scientific Institute in Milan, Italy. All patient samples were harvested in concomitance to routine diagnostic procedures and upon written informed consent approved by the San Raffaele Ethics Committee. Genomic DNA was extracted using the Qiamp Blood Minikit (QIAGEN, Hilden, Germany), checked for purity using a NanoDrop spectrophotometer (Thermo Scientific, Franklin, MA, USA) and stored at -20°C for further analyses. All relapses under analysis had been previously characterized and shown by SNP arrays (Illumina Human CNV370-Quad BeadArray or Human660W-Quad BeadChip, Illumina) or sequence-specific oligonucleotide hybridization HLA typing (INNO-LiPA, Fujirebio Inc.) to be classical or HLA loss [142 158 151].

3.3 NGS for high throughput detection of HLA loss relapse

3.3.1 NGS workflow for HLA loss detection

NGS based detection of HLA loss relapse follows a standard NGS workflow with PCR reactions targeted to the different HLA loci, as described in Lange et al. *BMC* 2013 [159]. The four main steps 1) DNA isolation and quantification, 2) Library preparation and quantification, 3) paired end sequencing on a MiSeq Illumina platform, 4) Bioinformatical data analysis are outlined in Figure 3.1 and described in the following.

3.3.1.1 DNA isolation and quantification

Genomic DNA extraction and quantification was performed as described in paragraph 3.2.2.

3.3.1.2 Library preparation and quantification

Library preparation followed the concept of two-step-nested dual indexing PCR as described in Lange et al. and outlined also in Supplementary Table 9.2[159]. Briefly, a first locus-specific PCR amplification of HLA-A,B,C,DRB1,DQB1,DPB1 exons 2-3 was performed separately for each locus, using 20 ng of input genomic DNA each, using primers provided by the DKMS Life Science lab (Dresden, Germany). The six individual PCR products of a given sample were pooled to reach equimolar concentrations for each locus. Subsequently, a second index-PCR amplification was performed on the pooled amplicon template of each sample, using primers containing both the barcodes (sample identifiers) and the sequencing adapter for the Illumina flow cell. Both PCRs were performed using Roche High Fidelity Fast Start Kit (Roche, Basel, Switzerland). Finally, library preparation was completed by pooling the entire index PCR products of all samples. Library purification was performed using magnetic beads AMPure XP (Beckman Coulter, Brea, USA) with a ratio of 0.7:1 beads to DNA, and quantified by Qubit fluorometric quantitation (Invitrogen, Thermo Scientific, Franklin, MA, USA). All pipetting was done manually, without the help of a pipetting robot.

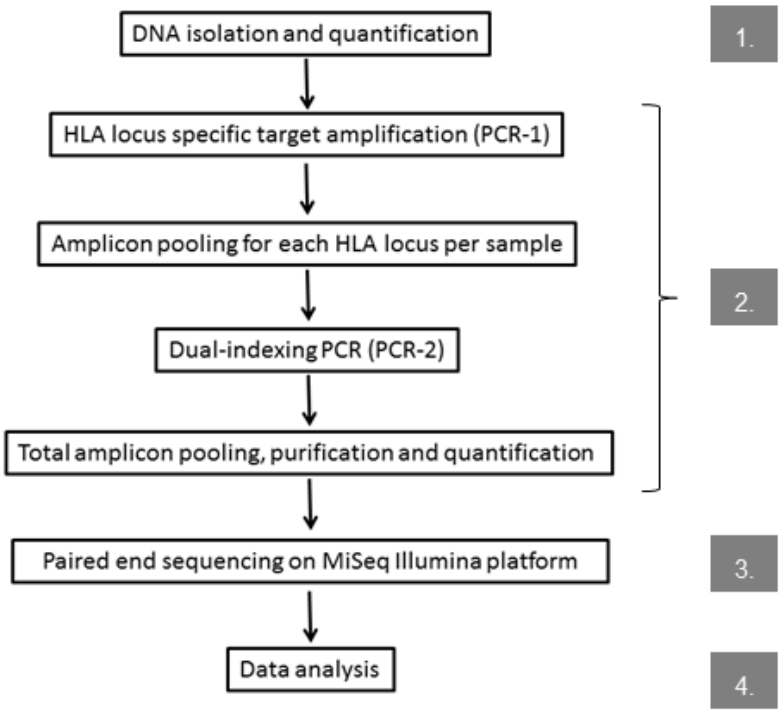


Figure 3.1: NGS workflow for HLA loss detection.

The workflow follows the 4 essential steps 1) DNA isolation and quantification, 2) Library preparation and quantification, 3) paired end sequencing on a MiSeq Illumina platform, 4) Bioinformatical data analysis, as described in Lange et al, BMC 2013.

3.3.1.3 Illumina paired end sequencing

Library amplicon pools were diluted 1:4000 in water and quantified by qPCR for flow-cell adapter sequences using the AriaMx Real-Time PCR System (Agilent, Santa Clara, CA, USA). Sequencing reagents were added as recommended by Illumina (MiSeq Reagent Kit V2 – Reagent Preparation Guide), in addition to spike-in of 20% phiX (single-stranded bacteriophage DNA) to increase sequence variability. Sequencing reactions were loaded at 7.5 pM on a MiSeq flowcell for paired end sequencing reaction at 251 cycles [159].

3.3.1.4 Bioinformatical data analysis

Raw data (FASTQ files) provided by the Illumina MiSeq system were analyzed by the DKMS proprietary NeXtype HLA typing Software, which is specifically adapted to paired-end amplicon sequencing. Briefly, NeXtype assigns HLA types on the basis of consecutive steps consisting in primer recognition, allele matching, result classification, exon combination, result rating, and user interaction.

HLA reference sequences were derived from the IMGT/HLA database [159]. For HLA chimerism analyses, any filter excluding allele calls with low read numbers were removed. Chimerism was assessed by direct counting of the read numbers for patient-specific alleles relative to donor-specific alleles, after subtraction of the non-specific read numbers in 100% donor and 100% patient DNA, respectively.

3.3.2 HLA typing validation by NGS

Technical validation of high resolution HLA typing was carried out on 2nd field HLA-A, B, DRB1, and DPB1 typing by the above-described NGS typing system according to Lange et al., *BMC* 2013. The average read-depth for HLA typing was 780 reads per base. HLA typing results were compared with those obtained on the same samples by standard intermediate or high resolution SSO and SSP performed at the Institute for Transfusion Medicine of the University Hospital Essen according to EFI accreditation standards. 22 HLA-typed control BLCL and 334 DNA samples from patients and their unrelated, 9-10/10 HLA-matched stem cell donors were included for validation. BLCL used in this study were purchased from ECACC, or established from PBMC samples of healthy donors using standard procedure [157].

3.3.3 HLA chimerism assessment by NGS

Sensitivity testing and HLA chimerism assessment was performed for each HLA locus by subjecting chimeric DNA to HLA typing according to the protocol described in paragraph 3.3.2. The average read depth for HLA chimerism assessment was however several-fold higher, i.e. at least 5000 reads per base.

For the validation, serial dilutions of genomic reference DNA extracted from PBMC of unrelated healthy individuals with different HLA typings (artificial chimeric DNA) were generated at 100%, 60%, 30%, 10%, 5%, 1%, and 0.1% (Table 4.8). For the analysis of HLA loss relapses, DNA from relapse samples post-transplantation were analyzed in an analogous way.

3.3.4 Patients and donor samples

Thirty-four adult patients who experienced hematologic relapse (<5% morphologic blast counts) after receiving a first allogeneic HSCT mainly from unrelated donors for AML, ALL, or other malignancies between 2006 and 2016 at the University Hospital Essen, Germany, were included in the analysis. Enrollment was based on the presence of a positive (>5%) result in chimerism analysis by qPCR as described in paragraph 3.2.4, performed under EFI accreditation standards at the Institute for Transfusion Medicine of the University Hospital Essen, in at least one of the samples obtained at or less than 6 months prior to relapse and the availability of genomic DNA from that sample for 21 patients. The remaining 13 patients had a chimerism >5% evidenced by STR as described in paragraph 3.1.4 prior to relapse, with DNA available from the relevant sample. Moreover, all patients had at least one HLA mismatch in the graft versus host (GvH) direction (i.e. an HLA allele present in the patient but absent in the donor). Patient and donor characteristics, including diagnosis, HLA matching status, time to relapse and percent chimerism outside HLA and inside HLA by NGS are shown in Table 4.8 and 4.9 (see pages 74 and 76). All samples were harvested in concomitance with routine diagnostic procedures and upon written informed consent under clinical protocols approved by the Ethical Review Board of the University Hospital Essen, in accordance with the Declaration of Helsinki.

4. Results

4.1 Clinical utility of qPCR chimerism and engraftment monitoring

Although qPCR has been explored for hematopoietic chimerism (HC) monitoring after allo-HSCT, evidence regarding its clinical utility compared with standard STR is still limited. Here we comparatively studied commercial STR and qPCR chimerism for the follow-up of allo-HSCT, and the association with clinical outcome endpoints.

4.1.1 HSCT characteristics and outcome

The study included 30 adult patients who received a first allo-HSCT for the cure of mostly AML and ALL at the University Hospital Essen between 2006 and 2013, and had a total of 459 retrospective longitudinal follow-up samples available. Patient, donor and transplant characteristics are shown in Table 4.1. Enrolment criteria were the availability of genomic DNA from at least 5 follow-up samples post-transplantation with the respective STR chimerism result, and the first transplantation from an HLA matched (mostly MUD) donor for a hematologic malignancy. Monitoring of tumor-specific molecular MRD markers could not be systematically included in the analysis, because it was performed only on sporadical samples, but was used to help in the outcome analyses for the clinical endpoint relapse where possible (see below). Transplant characteristics were homogenous for conditioning regimen and GvHD prophylaxis except for the use of ATG, which was administered in 19 of 30 patients.

The HSCT outcome characteristics as well as information on the longitudinal follow-up samples analyzed are presented in Table 4.2. The median time of follow-up was 1504 (317-2981) days. At last follow-up, 20 patients were alive and in complete remission, whereas the remaining 10 patients had died of non-relapse mortality or relapse.

Table 4.1: Patient, donor and transplant characteristics.

UPN #	Disease Diagnosis, Stage	Don Type, HLA Matching	Pt-Don Sex	Pt-Don CMV Serostatus	Conditioning Regimen	Date HSCT
2475	AML M2, CR1,	MUD 9/10	M - M	neg - pos	MA[a,c]	09.10.2007
2696	ALL (pre-T), CR1	MUD 10/10	M - M	pos – neg	MA[b,c]	20.01.2009
2765	ALL (pre-T), CR1	MUD 10/10	F - F	pos - neg	MA[b,d]	09.06.2009
2819	AML M0–MDS, persistent	MUD 10/10	M - M	neg - neg	MA[b,c]	03.10.2009
2920	AML M2, CR2	MUD 10/10	F - F	neg – neg	MA[b,c]	04.05.2010
2951	AML M2, CR1	MUD 10/10	M -M	pos – neg	MA[b,c]	02.07.2010
2984	MDS, persistent	MUD 9/10	M - M	neg – neg	MA[b,c]	13.09.2010
2988	AML M2, CR1	MUD 10/10	F - F	neg – neg	MA[b,c]	16.09.2010
3031	AML M2, CR1	MUD 9/10	M - M	neg – neg	MA[a,d]	25.11.2010
3052	AML M4, CR2	MUD 9/10	F - F	neg – pos	MA[b,c]	04.01.2011
3072	ALL, CR>2	MUD 10/10	M - M	pos –neg	MA[b,d]	17.02.2011
3119	AML, CR1	MUD 10/10	F - F	neg – pos	MA[b,c]	26.05.2011
3149	AML M4, Relapse	MUD 10/10	M - M	pos – neg	MA[b,d]	25.07.2011
3351	AML M2, CR1	MUD 10/10	M - M	neg – pos	MA[a,c]	21.08.2012
2435	AML M5b, PR2	MUD 10/10	M - M	neg – neg	MA[a,d]	11.07.2007
2909	AML M2, CR2	MUD 10/10	F - F	pos – neg	RIC	13.04.2010
2942	AML, CR1	MUD 10/10	M - M	pos – neg	MA[a,d]	22.06.2010
3222	AML M6, CR1	MUD 10/10	M - M	pos – pos	MA[a,d]	28.11.2011
2738	AML M6, CR1	MUD 9/10	M - M	neg – pos	MA[a,c]	15.04.2009
2848	AML M1, CR1	MUD 10/10	M - M	neg – neg	RIC	31.11.2009
3457	AML M4, CR1	MUD 10/10	F - F	pos – neg	MA[b,c]	25.03.2013
2265	AML M0, CR1	MUD 10/10	M - M	neg – neg	MA[b,c]	28.06.2006
2304	ALL, CR1	MUD 10/10	M -M	pos – neg	MA[a,c]	19.06.2006
2314	AML M0, CR1	MUD 9/10	M - M	neg – neg	MA[b,d]	06.10.2006
2322	AML, CR1	HLA-id. sib	F -F	neg – pos	MA[a,d]	26.10.2006
2338	AML M2, PR1	MUD 10/10	F - F	pos – neg	MA[b,d]	27.112006
2857	ALL, persistent	MUD 10/10	F - F	pos – neg	MA[a,d]	22.12.2009
3392	AML M5b, CR2	MUD 9/10	M - M	neg - pos	MA[a,c]	06.11.2012
3422	CMML, persistent	MUD 10/10	F - F	neg - pos	MA[b,c]	17.01.2013
3462	AML M3, CR2	MUD 9/10	M - M	neg - pos	MA[a,c]	03.04.2013

All patients received unmanipulated PBSC. RIC was based on Fludarabin, Busulfan and ATG pre-HSCT. MA conditioning was based on different agents [a]with or [b]without TBI, and [c]with or [d]without ATG as part of GvHD prophylaxis, which was based on cyclosporine A and methotrexate for all unrelated HSCT, and none for HLA-identical sibling HSCT.

Table 4.2: HSCT outcome characteristics and longitudinal follow-up samples.

UPN #	Engraftment[a]	Relapse[b]	DLI[c]	CMV reactivation[d]	aGvHD[e]	Survival status[f]	Follow-up d	Samples d (N)
2475	d19, normal	No	No	d56	No	alive CR	2578	30 – 1458 (17)
2696	d21, normal	No	No	No	No	alive CR	2135	97 – 1204 (17)
2765	d11, normal	No	No	No	No	alive CR	2072	22 – 1408 (18)
2819	d20, normal	No	No	No	No	alive CR	1879	26 – 828 (18)
2920	d18, normal	d324	d380 - 436 [g]	No	No	d795, NRM	795	30 – 723 (21)
2951	d18, normal	d829	d714 – 784[h]	No	No	d888, Relapse	888	32 – 829 (25)
2984	d13, normal	No	No	No	No	alive CR	1579	28 – 868 (18)
2988	d17, normal	No	No	No	No	alive CR	1526	31 – 718 (18)
3031	d13, normal	No	No	No	II-III, d33	alive CR	1534	21 – 1149 (13)
3052	d13, normal	No	No	d56	II-III, <d31	alive CR	1491	28 – 839 (16)
3072	d8, normal	No	No	d34	II, <d27	d731 NRM	731	21 – 697 (14)
3119	d13, normal	d510	d536 – 550[g]	No	No	d696, Relapse	696	46 – 628 (18)
3149	d11, normal	d633[i]	No	No	No	alive CR	1290	23 – 633 (8)
3351	d15, normal	No	d443 – 528[j]	d36	No	alive CR	891	36 – 597 (16)
2435	d16, delayed	No	No	No	II-III, d30-60	alive CR	2743	75 – 596 (15)
2909	d14, delayed	d1592[i]	d1646 – 1771[g]	d50	No	alive CR	1771	92 – 1128 (14)
2942	d14, delayed	No	No	No	No	alive CR	1668	23 – 717 (18)
3222	d11, delayed	No	No	d50	No	alive CR	1156	22 – 785 (16)
2738	d28, PMC	No	d426 – 440[g]	d58	No	alive CR	2064	37 – 714 (26)
2848	d14, PMC	No	d354 – 430[g]	No	No	alive CR	1886	28 – 647 (35)
3457	d24, PMC	No	>d287[g]	No	No	alive CR	681	31 – 273 (8)
2265	d16, n.i.[l]	d1504[i]	d1589 – 1646[g]	No	No	alive CR	2981	1504 – 2302 (18)
2304	d23, n.i.[l]	No	No	d42	II, d29	d1313, NRM	1313	483 – 1270 (10)
2314	d13, n.i.[l]	No	No	d53	No	d1308, NRM	1308	467 – 1243 (7)
2322	d20, n.i.[l]	d1846, 2176	d1903	No	II, d21	d2297, NRM	2297	1475 – 2071 (11)
2338	d13, n.i.[l]	No	No	d49	No	alive CR	2891	309 – 1985 (10)
2857	d11, n.i.[l]	d464	d359 – 443[j]	No	No	d806, NRM	806	246 – 504 (13)
3392	d21, n.i.[m]	d104	None	No	No	d433, Relapse	433	30 – 219 (5)
3422	d17, n.i.[m]	d274	d355 – 549[g]	No	No	alive CR	741	27 – 441 (10)
3462	d15, n.i.[m]	d105[i]	No	No	No	d317, NRM	317	107 – 315 (6)

[a]Engraftment defined as day (d) post-HSCT in which WBC>500/µl were reached, followed by classification as normal, delayed, PMC or not informative (n.i.) as in Methods (paragraph 3.1.2). [b]day of hematological or cytogenetic (UPN#3422) relapse, if applicable. [c]day of DLI, if applicable. [d]day of reactivation in the first 100d post-SCT if applicable. [e]Grade II-IV, followed by d post-HSCT if applicable. [f]day of death post-HSCT if applicable, followed by cause of death. [g]DLI for treatment of relapse. [h]DLI for decreasing CD34+ HC in STR. [i]no HC samples before relapse. [j]DLI for MRD positivity. [k]DLI for PMC in STR. [l]no samples <210d. [m]Relapse <210d

4.1.2 Comparative evaluation of HC results obtained by STR and qPCR

459 samples (364 PB and 95 BM) were available for comparative HC analysis by STR (performed as part of the follow-up routine diagnostics) qPCR (performed ad hoc on frozen DNA samples), as described in Methods (paragraph 3.1.4 and 3.1.5). These samples had been obtained between days 21 and 2302 post-HSCT, with at least 5 follow-up samples available for each patient (Table 4.2).

For each patient, we determined at least two chimerism qPCR markers informative for engraftment and relapse, i.e. markers positive in the patient but negative in the donor. The median number of informative qPCR markers in the entire cohort was 7 (minimum 3 – maximum 11) (Figure 4.1A). The final HC value was determined as mean values between the 2 selected assays, preferentially located on 2 different chromosomes. According to their documented sensitivity [57-59,63], the thresholds of >1% for STR and <0.1% for qPCR were used as respective cut-offs for positivity or negativity of HC analysis by the two methods. Based on this, the 2 methods had an overall concordance of $R^2 = 0.94$ (Figure 4.1B), in line with previous reports [57,64,65]. In particular, 64 samples (13.9%) were concordantly positive and 301 samples (65.6%) concordantly negative in STR and qPCR, for an overall concordance of 365 of 459 samples (79.5%) between the methods (Figure 4.1B and Table 4.3).

To address the question whether the sample source had an influence on the results, 44 samples drawn in parallel from BM and PB from the same patient were analyzed by qPCR chimerism. The results showed a high overall concordance of $R^2 = 0.98$ between the two sources (Figure 4.1C). However, the concordance rate between STR and qPCR was higher in PB (84%) than in BM (62%) samples (Table 4.3).

In the total of 459 samples analyzed, 94 (20.5%) showed discordant results between STR and qPCR; these included 58 of 364 PB (16%) and 36 of 95 BM samples (38%). In all cases discordance was due to a negative result in STR and a positive result in qPCR (Table 4.3). A closer analysis of the 94 discordant samples revealed that 41 of them (12 BM and 29 PB) were from patients with engraftment abnormalities, as described below, and another 42 of them (14 BM and 28 PB) were from patients before or after post-transplant relapse (Table 4.4). Only 11 discordant samples (10 BM and 1 PB) were found in patients with normal engraftment kinetics and without relapse, in particular in the early post-transplant period (Table 4.4).

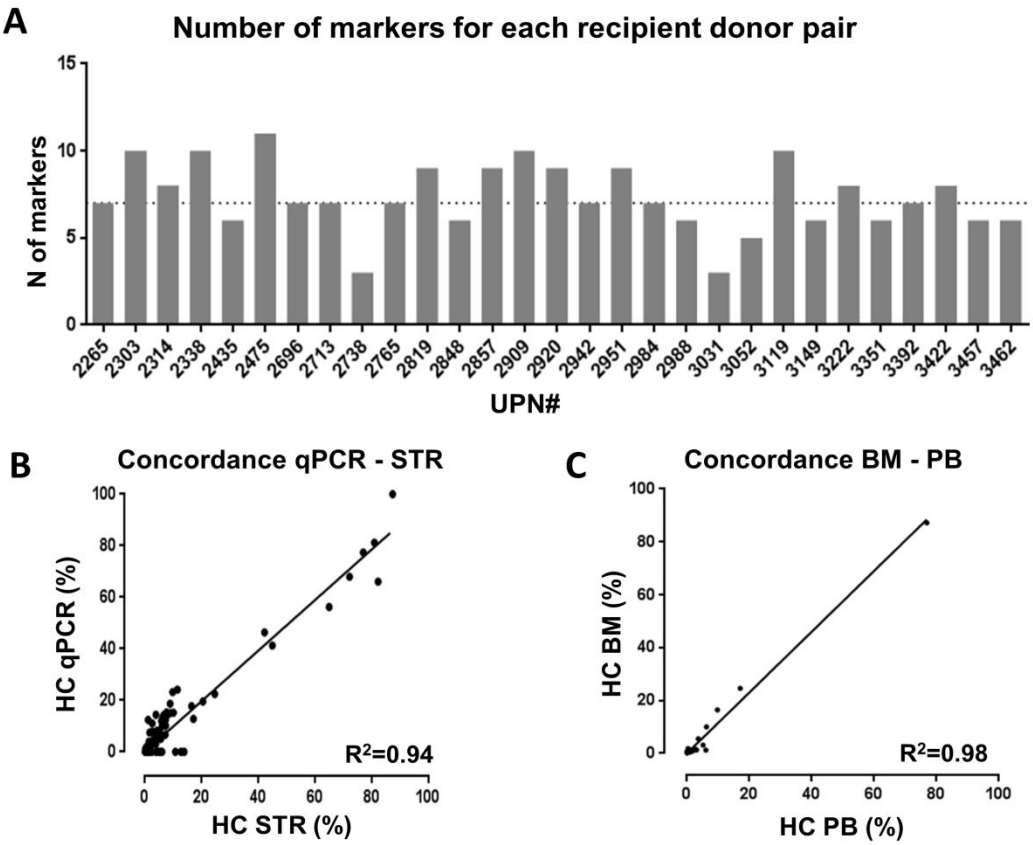


Figure 4.1: Number of informative qPCR markers and concordance ratesness of the samples according to the chimerism method or the sample source.

(A) Number of informative markers (i.e. positive in the patient and negative in the donor) identified in each of the 30 patient-donor pairs analyzed. The mean number of informative markers was 7 (3-11). (B) High overall concordance between absolute percentage of chimerism obtained by qPCR and STR in the459 samples studied. (C) High concordance between absolute percentage of chimerism obtained by qPCR in 44 samples drawn in parallel from BM and PB.

Table 4.3: Concordant and discordant HC results by STR and qPCR.

Method Result	PB N (%)[a]	BM N (%)[a]	Total N (%)[b]
STR pos / qPCR pos	**48 (13)**	**16 (17)**	64 (13.9)
STR neg / qPCR neg	**258 (71)**	**43 (45)**	301 (65.6)
Total Concordant	306 (84)	59 (62)	365 (79.5)
STR pos / qPCR neg	**0 (0)**	**0 (0)**	0 (0)
STR neg / qPCR pos	**58 (16)**	**36 (38)**	94 (20.5)
Total Discordant	58 (16)	36 (38)	94 (20.5)
Total Overall	364 (100)	95 (100)	459 (100)

[a]Percentages refer to the total overall number of PB or BM samples, respectively (last line in the column). [b]Percentages refer to the total overall number of samples (PB and BM combined) in the study (last line in the column).

Table 4.4: Characteristics of 94 discordant results between qPCR and STR.

Category	BM	PB	Total
Engraftment[a]			
Normal ≤210d post-HSCT	9	0	9
Normal >210d post-HSCT	1	1	2
Delayed ≤210d post-HSCT	5	12	17
Delayed >210d post-HSCT	0	0	0
PMC ≤210d post-HSCT	4	7	11
PMC >210d post-HSCT	3	10	13
Total Engraftment	**22**	**30**	**52**
Relapse[b]			
≤180d before relapse	6	21	27
>180d before relapse	4	1	5
after relapse	4	6	10
Total Relapse	**14**	**28**	**42**

[a]Engraftment: Normal, delayed or PMC as in Methods. [b]Relapse: hematologic or cytogenetic

4.1.3 Monitoring engraftment kinetics

All patients had engrafted with >500 WBCs/µL reached at a median time of 16 days (range, 8 to 28) post-SCT (Table 4.2). Nine patients were not informative for engraftment kinetics, either because no samples were available in the first 7 months after transplantation (6 patients) or because association of HC kinetics with engraftment was confounded by malignant disease relapse during that period (3 patients) (Table 4.2). In the remaining 21 informative patients, qPCR showed *normal* (sustained <0.1% patient chimerism) or *delayed* (sustained 0.1% to 1% patient chimerism) engraftment kinetics in 14 and 4 patients, respectively, whereas 3 patients presented with *PMC* (sustained >1% chimerism) by STR and qPCR. In patients with *normal* engraftment, in the first 7 months post-transplant, of 88 informative samples, 1 (1.1%) was positive in STR and 11 (12.5%) was positive in qPCR.

The single positive sample in STR was concordantly positive also in qPCR and occurred in a BM from patient 2920 at day 177 post-HSCT, with relapse presenting 147 days later at day 324 (ie, beyond the first 7 months post-transplant) (Table 4.2). The same patient had a positive PB sample in qPCR at day 220 post-HSCT (ie, 104 days before relapse). The remaining 9 positive qPCR samples from this period in the *normal* engraftment patients were exclusively from BM (Figure 4.2A for 2 example patients). In patients with *delayed* engraftment, sustained low-level (0.1% to 1%) patient chimerism reached a peak in the first 4 months post-HSCT and spontaneously decreased thereafter, until conversion to full donor chimerism before the first 7 months post-transplant in all cases (Figure 4.2B for 2 example patients). Finally, *PMC* above 1% patient chimerism was evident in 3 patients by both STR and qPCR also beyond the first 7 months post-HSCT (Table 4.2). However, its onset and resolution by DLI was more accurately documented by qPCR than by STR (Figure 4.2C for 2 example patients). No correlations of engraftment abnormalities (ie, *delayed* engraftment or *PMC* with clinical outcome including acute GvHD, relapse or survival) were observed in our cohort (Table 4.2). However, this parameter appeared to be influenced by both donor–recipient CMV serostatus and CMV reactivation post-transplant.

4.1.4 Imprinting of CMV reactivation on engraftment and relapse

Ten of 30 patients in this study experienced CMV reactivation in the first 100 days post-HSCT (Table 4.2). This event was less frequent in 9 transplants of CMV-negative patients from a CMV-negative donor (1/10; 10%), compared with 21 transplants in which recipient and/or donor were CMV-positive (9/21; 42.8%), in accordance with previous observations [25]. We also confirmed previous results from us and others [56 29 33 30 31 32] showing a protective effect of early CMV reactivation on the post-transplant relapse incidence of AML. Of the 23 AML patients of this cohort, 8 experienced early CMV reactivation and 15 did not. Post-transplant relapse occurred in 1 of 8 AML patients (12.5%) with early CMV reactivation compared with 8 of 15 AML patients (53.3%) without (Table 4.2). In the 21 informative patients informative for engraftment kinetics, imprinting of recipient–donor CMV serostatus and CMV reactivation on this parameter was monitored.

Delayed engraftment or PMC was observed in 3 of 8 informative seropositive patients (37.5%) transplanted from a seronegative donor, compared with 1 of 5 informative seronegative patients (20%) transplanted from a seropositive donor (Table 4.2). Likewise, delayed engraftment in qPCR or PMC occurred in 3 of 7 informative patients (42.8%) with early CMV reactivation post-transplant, compared with 4 of 14 (28.5%) without. In 1 patient (patient 2738) CMV reactivation at day 58 post-transplant closely preceded the onset of PMC documented by qPCR at day 110 (Table 4.2 and Figure 4.2C).

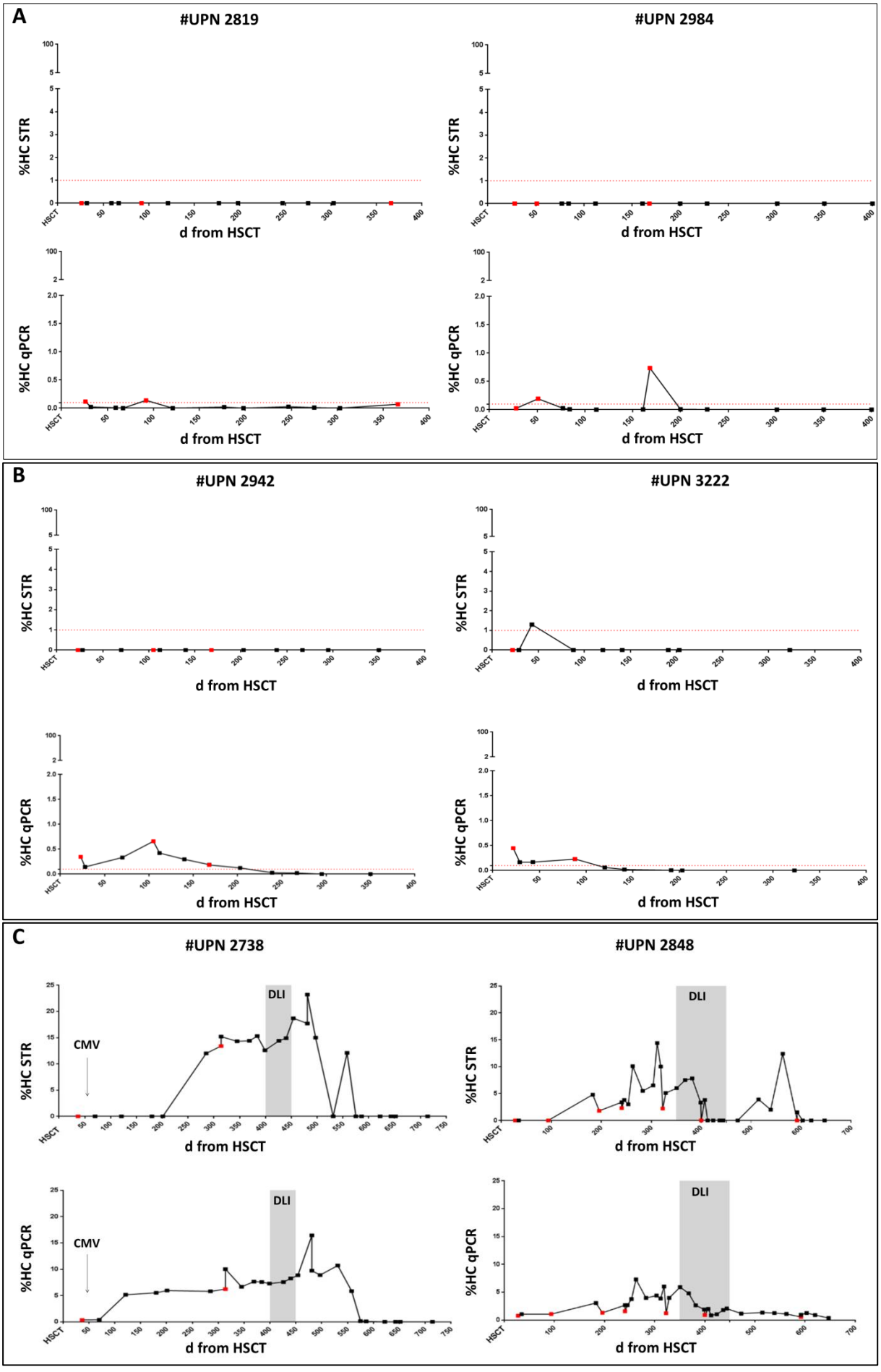
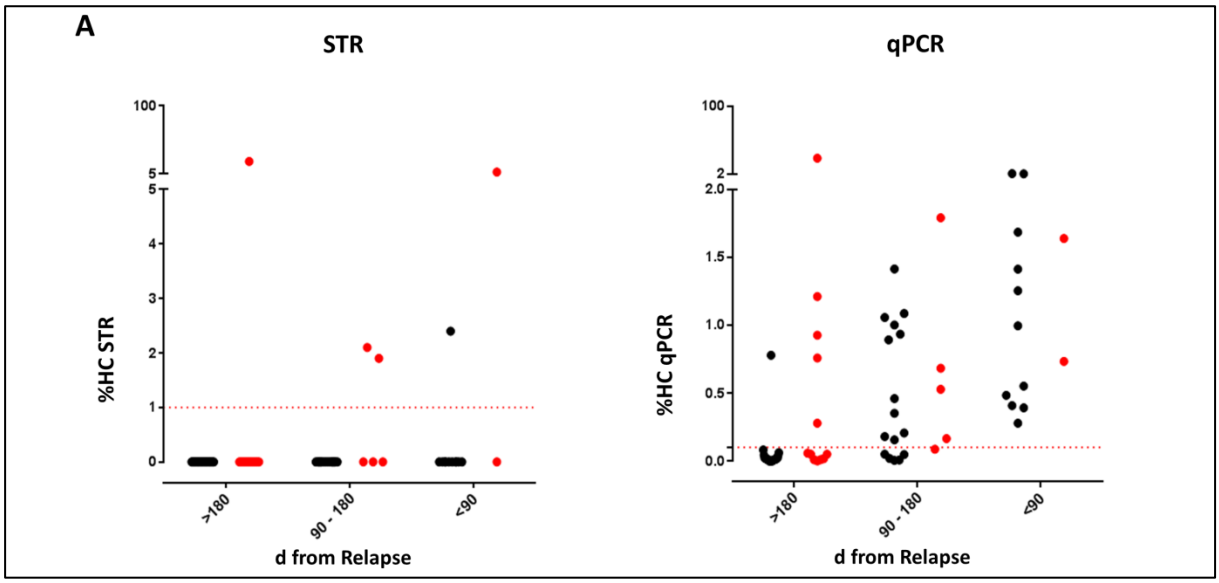

A
#UPN 2819
#UPN 2984
%HC STR
%HC qPCR
d from HSCT
B
#UPN 2942
#UPN 3222
%HC STR
%HC qPCR
d from HSCT
C
#UPN 2738
#UPN 2848
DLI
CMV
%HC STR
%HC qPCR
d from HSCT

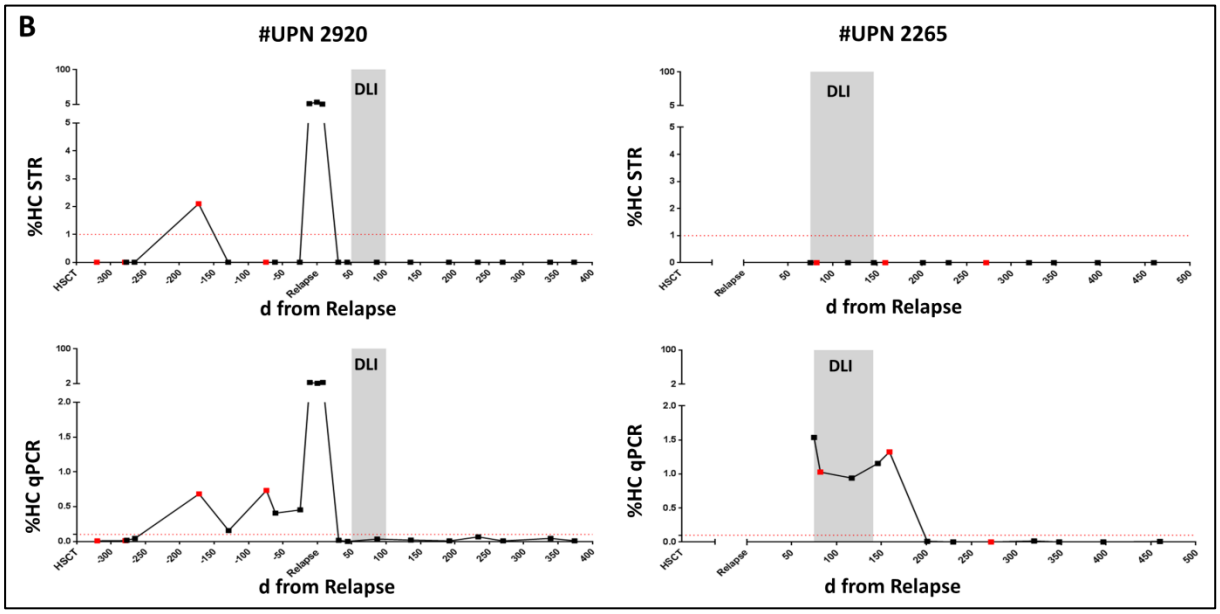
Figure 4.2: Kinetics of engraftment monitored by STR and qPCR.

Shown are HC results obtained by STR (top) or qPCR (bottom) on longitudinal follow-up samples of example patients (Table 4.2). BM and PB samples are indicated as red and black dots, respectively. The HC positivity threshold of 1% and 0.1% for STR and qPCR, respectively, is indicated as dotted red line in the relevant plots in A and B. (A) Normal engraftment kinetics in two example patients. Note that isolated positive results in qPCR were obtained only on BM samples in the early post-HSCT period. (B) Delayed engraftment kinetics in two example patients. Note that in both patients, decreasing mixed chimerism was observed as of day 100 post-HSCT. (C) PMC in two example patients. Both patients received DLI (shaded area), with reversion to full donor HC within 196 and 296 days, respectively, as documented by qPCR. In one patient (patient 2738, left panels), PMC onset was closely preceded by CMV reactivation (arrow).

4.1.5 Association between qPCR chimerism monitoring and relapse

Twelve relapses of malignant hematologic disease had occurred post-transplant in 11 patients under study, with 1 patient suffering 2 consecutive relapses (Table 4.2). For 4 of these 12 relapses, no follow-up samples were available within the 6 months before onset. For the remaining 8 informative relapses, 1 to 9 samples were available in the 6 months before onset, for a total of 33 pre-relapse samples (6 BM and 27 PB). Of these, 6 of 6 BM samples (100%) and 23 of 27 PB samples (85.2%) were positive by qPCR until day 180 pre-relapse, compared with 3 of 6 BM samples (50%) and 1 of 27 PB samples (3.7%) by STR. For comparison, 1 of 26 PB samples and 5 of 12 BM samples were positive by qPCR in the same patients at time points earlier than day 180 pre-relapse and 0 of 26 PB samples and 1 of 12 BM samples in STR (Figure 4.3A). In total, 8 of 8 relapses (100%) were predicted by at least 1 positive qPCR result in either PB or BM in the 6 months before onset, compared with 3 of 8 relapses (37.5%) by STR on BM and 1 of 8 (12.5%) on PB. Of note, in all patients with several consecutive samples available for analysis in the 6 months before relapse, repeated positivity was seen by qPCR both on BM and PB, whereas the positive STR cases were isolated and mainly restricted to BM (Figure 4.3B and 4.3C left, for 4 example patients).


A
STR
qPCR
%HC STR
%HC qPCR
>180
90 - 180
<90
d from Relapse


B
#UPN 2920
#UPN 2265
DLI
%HC STR
%HC qPCR
HSCT
Relapse
d from Relapse

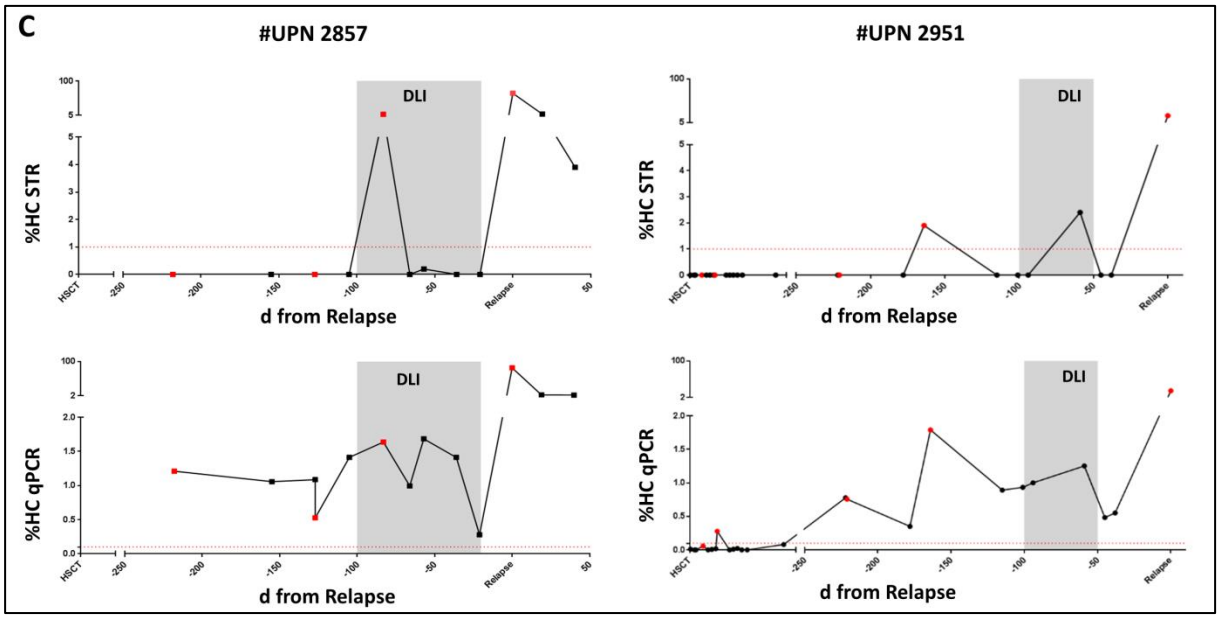

C
#UPN 2857
#UPN 2951
DLI
%HC STR
%HC qPCR
HSCT
Relapse
d from Relapse

Figure 4.3: HC kinetics of STR and qPCR in relapse patients.

BM samples are indicated as red and black dots, respectively. The HC positivity threshold of 1% and 0.1% for STR and qPCR, respectively, is indicated as dotted red line in the relevant plots. (A) STR (left) and qPCR (right) HC results on a total of 71 (53 PB, 18 BM) samples from 8 informative relapses. Thirty-three of these samples were drawn in the 6 months pre-relapse (11 PB and 2 BM ≤ day 90, 16 PB and 4 BM between days 90 and 180), and the remaining 38 samples (26 PB and 12 BM) more than 180 days before relapse onset. Note the striking increase in positive qPCR on PB in samples ≤ 180 days (23/27) compared with >180 days (1/26) before relapse. (B) Longitudinal HC follow-up of relapse treatment by chemotherapy and DLI in two example patients (Table 4.2) by STR (top) or qPCR (bottom). In patient 2920, samples were available also pre-relapse and showed sustained positivity of qPCR but not STR in the 6 months before relapse. In this patient, consolidation of remission by DLI after post-relapse chemotherapy was demonstrated by full donor HC also in qPCR. For patient 2265, samples were available only after induction of remission by chemotherapy and showed low levels of mixed HC in qPCR but not STR, which converted to full donor HC 126 days after DLI. (C) Longitudinal HC follow-up in the 6 months before relapse in two example patients (Table 4.2) by STR (top) or qPCR (bottom). Note that relapse was predicted by sustained positivity in qPCR both on BM and PB, whereas positivity in STR pre-relapse was sporadic and mainly in BM. Both patients received DLIs to prevent relapse based on positivity in MRD or STR on sorted CD34+ cells (not shown). The inefficacy of DLI to prevent relapse was shown by lack of reversion to full donor chimerism pre-relapse by qPCR but not by STR in both patients.

4.1.6 qPCR chimerism monitoring and donor lymphocyte infusions

A total of 12 patients received donor lymphocyte infusions (DLI) post-transplantation for prevention or treatment of PMC or relapse (Table 4.2). In two PMC patients (patients 2738 and 2848), conversion from mixed to full donor chimerism after DLI could be monitored and was shown by qPCR to proceed gradually over a period of 196 and 296 days, respectively, whereas the kinetics of HC decline was less evident in STR analysis (Figure 4.3B). In 2 patients receiving DLIs due to sporadic positivity in MRD or STR on sorted CD34+ cells (data not shown), DLI was inefficient in preventing relapse, and this was correlated with sustained low-level positivity in qPCR but not STR until relapse (Figure 4.3C). Finally, in 2 patients with informative samples after post-relapse DLI, qPCR demonstrated efficient consolidation of chemotherapy-induced remission in 1 patient (Figure 4.3B, left), whereas conversion to full donor chimerism took 126 days from the start of DLI in the other patient (Figure 4.3B, right).

4.2 HLA loss diagnosis by a new qPCR approach

4.2.1 Rationale of HLA chimerism

Since qPCR is a highly sensitive methodology for chimerism and in particular relapse monitoring after HSCT (see section 4.1 and paragraph 4.1.5), we set out to use this method to overcome the difficulties related to the detection of HLA loss relapses. As outlined in the introduction (paragraph 1.4.3), these particular immune escape variants after partially HLA-mismatched HSCT are identified through the combined application of totally different methodologies to assess chimerism for polymorphic markers located outside the HLA system by STR or qPCR on the one hand, and the percentage of patient-specific HLA by standard HLA typing methodologies or SNP arrays on the other [142,145,151,158]. This is a severe limitation since the applied methods have different and generally not elevated sensitivities of around 5%, precluding the diagnosis of HLA loss prior to overt hematologic relapse. Moreover, the combined interpretation of these methods is not trivial and is not amenable to the clinical routine, but is confined to highly specialized research laboratories.

We reasoned that these difficulties could be circumvented by the concomitant targeting of qPCR chimerism to polymorphisms outside and inside the HLA system. For outside HLA polymorphisms, we used a commercial qPCR kit targeting insertion-deletion polymorphism as described in the Methods section (paragraph 3.2.4). qPCR reactions for Inside HLA polymorphisms were designed *ad hoc* for the most frequent HLA allele groups (HLA markers), as described below. Because the large majority of post-transplant relapses are of patient origin, they will consequently be positive for the patient-specific non-HLA marker. In contrast, while “classical” (ie, non-HLA loss) relapses will be concordantly positive also for HLA markers, HLA loss relapses will be positive for non-HLA markers and negative for patient-specific HLA markers, because of their selective genomic loss. The comparative evaluation of HC with HLA and non-HLA markers therefore represents an easy tool to discriminate classical from HLA loss relapses (Figure 4.4).

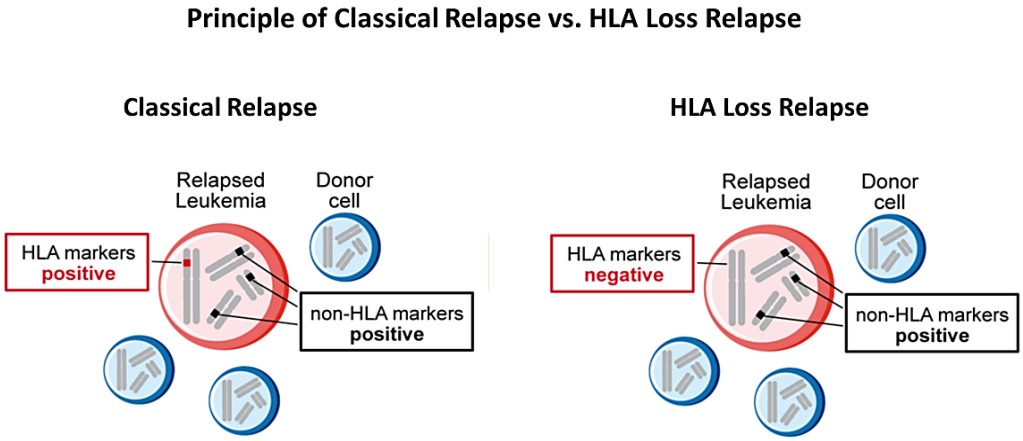


Figure 4.4: Schematic representation of the rationale underlying HLA qPCR markers.

Shown is the simultaneous presence of leukemic blasts (in red) and normal donor hematopoietic cells (in blue) in a typical sample of a patient with relapse after HSCT. In each cell, 4 representative chromosomes are depicted, with a patient-specific HLA marker (in red) and 3 hypothetical non-HLA markers (in black). Leukemic blasts in the classical relapse will be positive for both the HLA and the non-HLA markers (left). Leukemic blasts in the HLA loss relapse will be positive for the non-HLA markers but negative for the HLA markers because of a selective genomic loss of mismatched HLA as an immune escape mechanism of leukemia relapse (right).

4.2.2 Design and development of HLA allele specific qPCR assays

Due to the extremely high degree of polymporhism displayed by HLA genes, the design of qPCR reactions comprehensively covering all or even most alleles and loci is a near to impossible task. We therefore focused on 27 HLA-A, HLA-C, and HLA-DPB1 allele groups, selected based on their frequency in different ethnic populations (Table 4.5) [160]. These 3 loci were chosen because they are often mismatched both in haploidentical and in MUD HSCT, but also in MUD HSCT, the two most frequent donor sources to date (Figure 1.14, paragraph 1.3.6). For the design of qPCR reactions targeting these allele groups, the genomic nucleotide sequences of HLA-A, -C and -DPB1 alleles were aligned and primer design was carried out as described in section 3.2.1. Each primer was selected to discriminate specific SNPs in the exon or intron of the selected HLA allele group at their 3' end. The HLA allele groups targeted by each assay are listed in Table 4.5. Using this qPCR panel, at least one informative reaction (ie, targeting at least one HLA-A, HLA-C, or HLA-DPB1 allele present in the patient and absent in the donor) was found with a frequency of 70.3% and 66.4%, respectively, in a series of 454 haplo- HSCT and 113 UD-HSCT with a mismatch in the GvH direction at either of the three loci (Table 4.6).

Table 4.5: HLA-A, C and DPB1 frequencies in worldwide populations.

			Allele Frequencies (%)[1] in defined ethnic groups					
HLA Locus	**Reaction Name**	**Target Alleles**	**European Caucasian[2]**	**USA Caucasian[3]**	**Black-African American[4]**	**Hispanic[5]**	**Japanese[6]**	**Chinese[7]**
A*	**KMR501-A**	01	15.15	16.48	5.1	7.41	0.4	2.4
		36	0	0.01	2.5	0.48	-	0
	KMR502-A	02	28.26	28.87	18	27.08	24.73	22.6
	KMR504-A	11	5.66	6.09	1.4	4.56	9.36	22.6
	KMR505-A	23	2.32	1.97	11	3.69	-	0.2
		24	9.84	8.66	2.6	13.87	37.31	17.3
	KMR506-A	25	2.39	2.1	0.3	1.01	-	0.01
		26	3.71	3.19	1.5	3.04	11.54	2.6
		34	0.06	0.09	3.4	0.48	0.01	0.05
		66	0	0.41	2.5	0.74	0.01	0.04
		68	4.29	4.04	10	9.24	0.01	0.5
		69	0.09	0.15	-	0.28	-	0.7
C*	**KMR511-C**	03	12.97	13.05	8.8	11.73	26.04	25.14
	KMR512-C	04	12.54	10.61	20.5	17.6	4.42	5.47
DPB1*	**KMR520-DPB1**	04:01	41.7	na	-	na	4.95	na
		23:01	0.3	na	-	na	-	na
		31:01	-	na	-	na	-	na
		34:01	0.1	na	-	na	-	na
	KMR521-DPB1	02:01	13.8	na	-	na	23.83	na
		04:02	10	na	-	na	9.83	na
		08:01	0	na	-	na	0.03	na
		105:01	-	na	-	na	0.03	na
		16:01	0.7	na	-	na	-	na
	KMR522-DPB1	01:01	5.6	na	-	na	0.03	na
		11:01	2.1	na	-	na	-	na
		13:01	2.4	na	-	na	1.79	na
		15:01	1.1	na	-	na	-	na

[1]Shown are the frequencies for 1st field HLA-A and C alleles, and 2nd field HLA-DPB1 alleles. Shown is the total number of allele copies in the population sample (Alleles/2n) in decimal format. Frequencies were obtained from the allelefrequencies.net database, selecting populations with at least 2000 reported alleles. [2]For HLA-A and C, a German population (N=39689) was used. For HLA-DPB1, an England Northwest population (N=2690) was used. [3]For HLA-A and C, a USA NMDP European Caucasia population (N=1242890) was used. No available data for HLA-DPB1. [4]For HLA-A and C, a USA NMDP African American population (N=416581) was used. For HLA-DPB1, an USA NMDP Black South or Central American population (N=4889) was used, in which none of the DPB1 targeted alleles were found (-). [5]For HLA-A and C, a USA NMDP Hispanic South or Central American population (N=146714) was used. No available data for HLADPB1. [6]For HLA-A and C, a Japanese population (N=18604) was used. For HLA-DPB1, a Japanese population (N=3078) was used. [7]For HLA-A, a Shanghai population (N=26266) was used. For HLA-C, a USA NMDP China population (N=99672) was used. No available data for HLA-DPB1. (na) no data available. (-) allele not reported in this population.

Table 4.6: HLA-specific qPCR assays developed in this study

HLA Target	Assay Name	Target HLA Allele Groups[1]	Efficiency in H_2O (%)[1,2,3]	Efficiency in chimeric conditions (%)[1,2,3]	Sensitivity (%)[1,3]	Informative HSCT (%)[4]	
						Haploidentical (n=454)	Unrelated (n=113)
A*	KMR501-A	01, 36	87.7	86.1	0.03	10.4	0
	KMR502-A	02	87.6	90.6	0.03	12.1	0
	KMR504-A	11	94.4	90.7	0.16	3.5	0.8
	KMR505-A	23, 24	98.0	97.1	0.03	9.9	0.8
	KMR506-A	25, 26, 34, 66, 68, 69	94.3	91.0	0.16	11.2	4.4
C*	KMR511-C	03	90.8	90.8	0.03	3.1	0
	KMR512-C	04	84.5	86.1	0.16	10.6	0
DPB1*	KMR520-DPB1	04:01, 23:01, 31:01, 34:01	98.3	93.2	0.03	14.1	23.8
	KMR521-DPB1	02:01, 04:02, 08:01, 105:01, 16:01	100.8	92.1	0.03	17.2	31.8
	KMR522-DPB1	01:01, 11:01, 13:01, 15:01	96.8	94.7	0.03	3.1	16.8

[1]Efficiency and Sensitivity were tested on HLA-typed BLCLs obtained from the European Collection of Animal Cell Cultures or from healthy HLA-typed individuals. For assays targeting multiple HLA allele groups, the most frequent HLA allele group in worldwide populations[14] (Table 4.5) was used as target-positive reference DNA. Efficiency and Sensitivity were determined by serial dilutions of target-positive reference DNA in water or target negative DNA. [2]Reported percentages are the mean of at least 3 independent experiments performed at either of the 2 testing sites (OSR and UKE). Standard error of mean (SEM) was <4 in all cases. [3]Maximal reproducible sensitivity determined in at least 3 independent experiments performed at either of the 2 testing sites (OSR and UKE). [4]Percentage of informative HSCT based on HLA typing of patient and donors for 454 haplo-HSCT performed between the year 2000 and the year 2016 at OSR, and for 113 UD-HSCT performed in the year 2015 at UKE with at least one HLA-A, -C or -DPB1 mismatch in the graft-versus-host-direction. Some transplants had more than one informative reaction while others had none; the total percentage of transplants with at least one informative reaction was 71.6% and 66.4% for haplo-HSCT and UD-HSCT, respectively.

4.2.3 Technical validation of HLA qPCR assays

To validate their technical robustness and reproducibility, the HLA-specific reactions were extensively tested on different combinations of HLA-typed reference DNA, as described in the results (section 3.2).

On-target amplification efficiency averaged 92.8% (Table 4.6), with superimposable performance in water and in target-negative DNA (overall concordance by Pearson correlation $R^2 = 0.99$, $P < 0.0001$ (Figure 4.5).

Specificity and possible cross-reactivity testing was performed for each assay both *in silico* based on nucleotide sequences reported in the IMGT/HLA database, and in a panel of 45 positive and negative HLA-typed reference DNA (see paragraph 3.2.5 and Supplementary Table 9.1). Assays with multiple specificities were tested independently on positive reference DNA carrying each of the relevant HLA alleles or allele groups. The results of the specificity testings in Figure 4.6 show high specificity for the target HLA allele groups, although some weak but reproducible cross-reactivity (yellow squares in Figure 4.6) with other HLA alleles could be documented for some of the assays. Importantly, accuracy and precision in chimerism determination resulted very high for all reactions, with almost perfect concordance between the expected and experimentally determined quantification in serial dilutions of HLA target-positive into target-negative DNA (overall concordance by Pearson correlation $R^2 = 0.99$, $P < 0.0001$) (Figure 4.7). Maximal reproducible sensitivity defined according to minimum information for publication of quantitative real-time PCR experiments guidelines [156] was at least 0.16% for all reactions (Table 4.6).

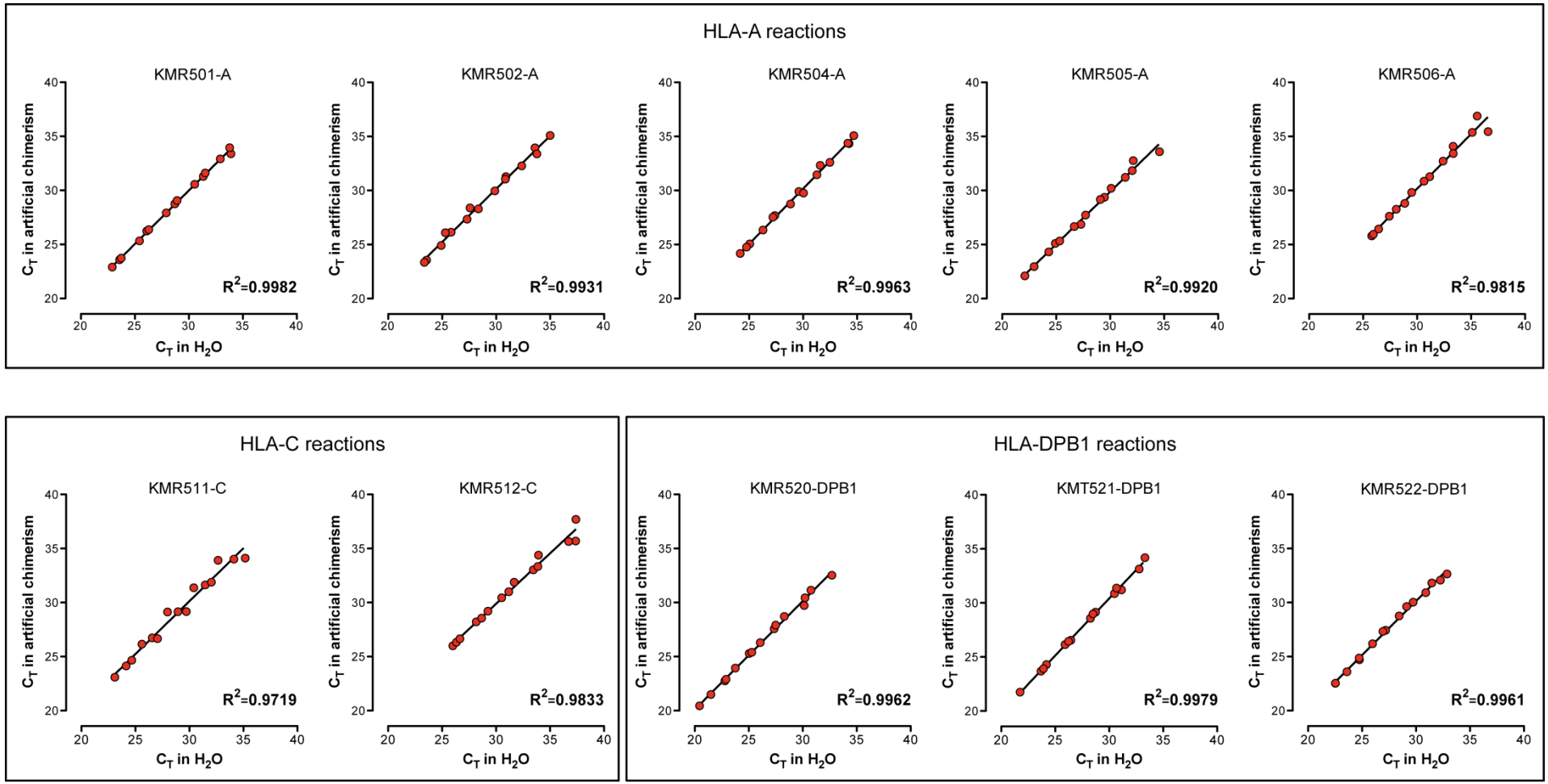


Figure 4.5: Correlation between results obtained from serial dilutions of target-positive DNA in water or in target-negative reference DNA.

Shown are the C_T values measured by serially diluting a target-positive genomic DNA into water (X-axis) or into target-negative DNA (Y-axis) for all 10 newly developed HLA marker-specific qPCR assays. Shown are results obtained from 3 independent experiments performed at the two sites (OSR and UKE), and under each plot is reported the Pearson correlation coefficient.

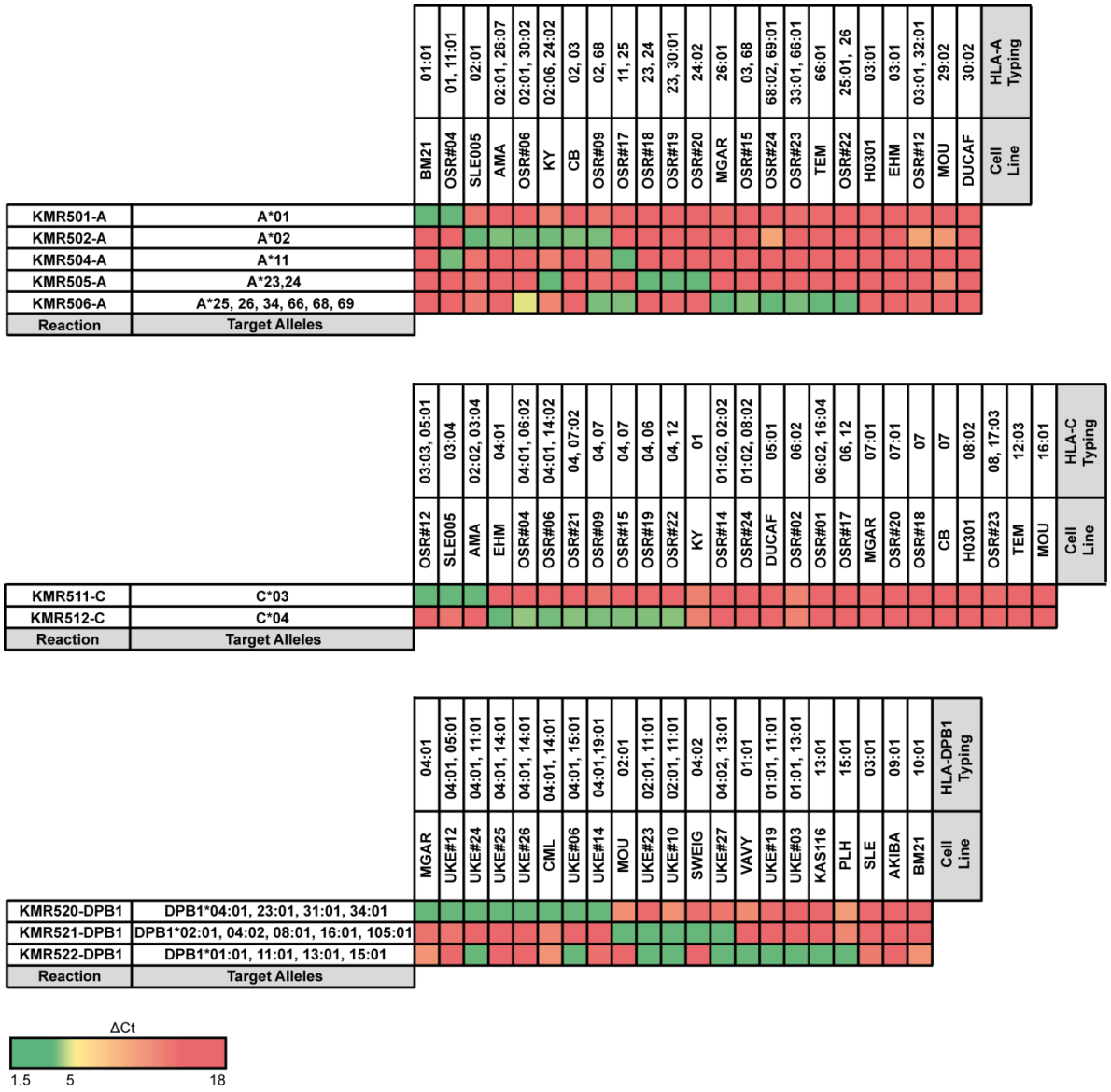


Figure 4.6: Specificity testing of the newly developed HLA marker-specific qPCR assays.

Tests were performed on 300ng of genomic DNA from the indicated reference panel. Shown is a heat-map displaying the ΔC_T values (C_T HLA marker - C_T reference) obtained upon testing each reaction against the panel of cell lines. Dark green and red indicate specific presence or absence of amplification, respectively, in the expected HLA allele groups. Cross-reactivity with HLA alleles outside the expected groups are indicated in orange-yellow.

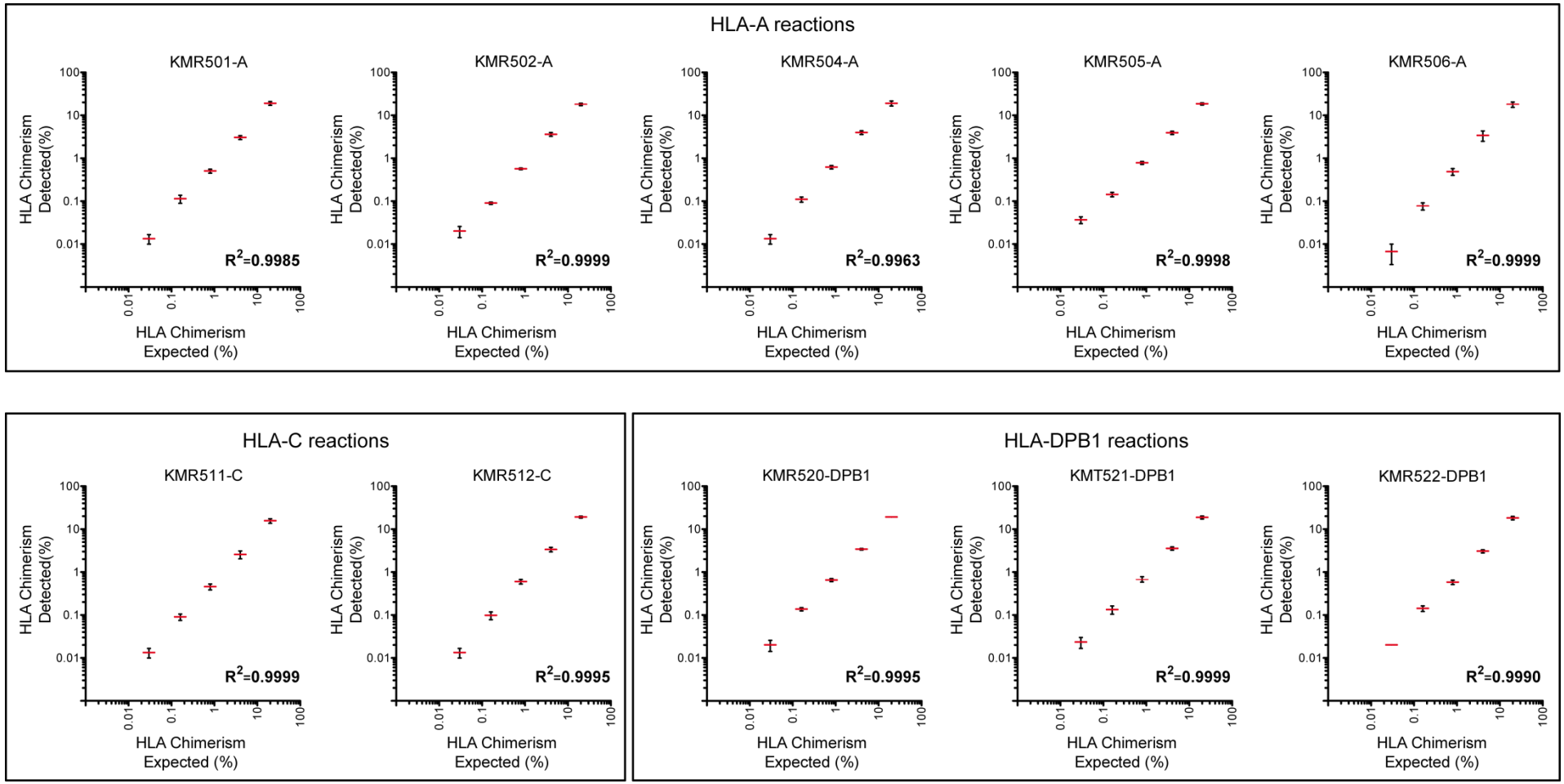


Figure 4.7: Correlation between the expected and the observed artificial chimerism results.

Shown is the correlation between the expected (X-axis) and experimentally measured (Y-axis) chimerism measured according to the $\Delta\Delta C_T$ method on positive reference DNA serially diluted into negative reference DNA. Results from 3 independent experiments are displayed as mean (red dashes) ±SEM (black whiskers), and under each plot is reported the Pearson correlation coefficient.

4.2.4 Clinical utility of the HLA markers

Clinical utility of the newly developed assays was validated on serial BM samples collected during the post-transplantation follow-up of 20 cases of leukemia relapse after haploidentical or MUD HSCT, as described in the Methods section (paragraph 3.2.6). These relapses had been previously shown by SNP arrays or SSOP HLA typing [142] [151,158] to be classical (N = 10) or HLA loss (N = 10). We reanalyzed these relapses by combining our newly developed HLA-specific assays with commercial qPCR chimerism assays for outside HLA insertion-deletion polymorphisms as described in the Methods (paragraph 3.2.4).

Data obtained for two representative patients who received haplo-identical HSCT and experienced a classical or an HLA loss relapse, respectively are shown in Figure 4.8A. Both cases showed concordant low (<3%) positivity for HLA and non-HLA markers in samples obtained prior to relapse, suggesting the detection of residual HLA-heterozygous host chimerism, in line with our previous data on qPCR chimerism monitoring (Results 4.1). At the time of classical relapse, positivity for both HLA and non-HLA markers rose to similar levels, both consistent with the blast percentage detected at BM examination (data not shown). In contrast, the HLA loss relapse sample showed positivity for the non-HLA marker that was again in line with the morphological blast percentage (data not shown), but as expected the inside HLA reaction marker remained negative (<1%).

In addition, HLA and non-HLA markers were quantified in parallel in 20 cases of leukemia relapse after haplo-HSCT or UD-HSCT which had been previously characterized to be classical (n=10, 9 after haplo-identical HSCT, 1 after UD-HSCT) or HLA loss (n=10, 9 after haplo-identical HSCT, 1 after UD-HSCT) (Figure 4.8B). Similar data were obtained for all 10 classical and 10 HLA loss relapse cases analyzed (Figure 4.8B). Because of the relatively low precision of qPCR in measuring high chimerism percentages, quantification of HLA and non-HLA markers in a given sample can have a result slightly different in classical relapses. HLA loss relapses can be unequivocally diagnosed only when HLA markers are negative (<3%, dashed line) and non-HLA markers are positive (>3%).

Of note, to cover the 20 cases, each of the 10 newly developed HLA-specific qPCR assays was employed at least once, documenting the relevance of developing multiple HLA markers and the valid coverage of our panel.

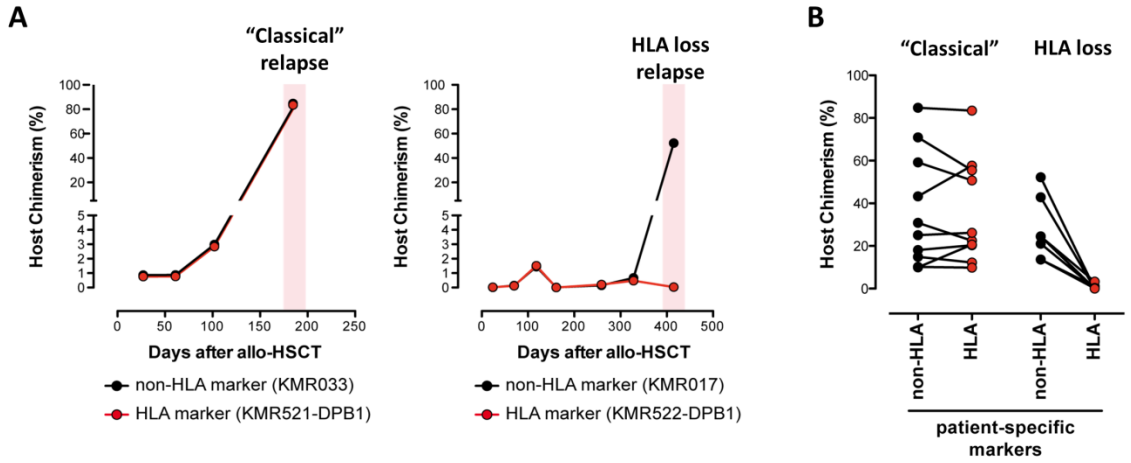


Figure 4.8: Clinical utility of HLA-KMR.

(A) Longitudinal post-transplant monitoring by HLA-KMR of a patient who experienced a classical relapse (time of relapse boxed in pink), alongside a patient-specific HLA marker (KMR521-DPB1 reaction, in red) and a patient-specific non-HLA marker (KMRtrack assay KMR033, in black). Note the concordance between the two assays at time of relapse, which identifies a classical relapse. Longitudinal post-transplant monitoring by HLA-KMR of a patient who experienced an HLA loss relapse (time of relapse boxed in pink), alongside a patient-specific HLA marker (KMR522-DPB1 reaction, in red) and a patient-specific non-HLA marker (KMRtrack assay KMR017, in black). Note the discordance between the two assays at time of relapse, which identifies a HLA loss relapse. (B) Summary of results obtained by chimerism quantification with HLA-KMR on 20 post-HSCT relapses (18 haploidentical, 2 unrelated). As expected, HLA markers (full panel of the newly developed HLA-specific reactions, red dots) and non-HLA markers (KMRtrack assays, black dots) yield concordant results in all classical relapses (left), and discordant results in all HLA loss relapses (right).

4.3 NGS based characterization of HLA immune escape

We set out to establish NGS based detection of HLA chimerism, taking advantage of a collaboration with the DKMS Life Science lab in Dresden, where HLA typing by NGS was pioneered several years ago (Lange et al. BMC 2013) and whose expertise in this specific field provided an important scientific and technological advantage. We first validated the NGS based HLA typing approach developed by DKMS in our laboratory, and then applied it to HLA chimerism detection using serial dilutions of DNA mixtures. Finally, we used the newly developed method to look for HLA loss relapses in a test cohort of patients from the University Hospital Essen who experienced relapse after MUD HSCT.

4.3.1 Validation of an NGS platform for HLA typing

Validation of NGS as platform for HLA typing was performed for the loci HLA-A, B, DRB1 on genomic DNA from 22 BLCL and 334 PBMC with known 2nd field HLA types (Table 9.3; samples described in section 3.3.3). Briefly, the polymorphic exons 2 and 3 of each gene were sequenced by NGS on an Illumina platform with an average read-depth of 780 reads per base, according to the protocols published by Lange et al. [159] and as described in the Methods section 3.3.2. HLA typing assignments were performed using the DKMS proprietary NeXtype software. The frequency of partial or complete drop-outs (no sequence reads available for analysis) was 2%. In the remaining 98% of pairs, concordance with the reported typing results was 100%. Univocal G-group or 2nd field resolution, almost never possible by conventional SSOP or SSP typing, was achieved in 94.7% of the HLA-A, B and DRB1 alleles by NGS. HLA-DPB1 typing was validated only on the 22 reference BLCL, since no typing information was available on the PBMC, and showed 100% concordance. Preferential amplification of one over the other allele present in heterozygous samples, resulting in a read distribution of the two alleles in a given sample other than the expected 1:1 ratio, was observed with variable frequency for HLA-A,B and DRB1. In contrast, for DPB1 the reads for the two alleles were generally close to a 1:1 ratio, with the only exception of samples involving DPB1*04:02 which showed lower read counts compared to the second allele in some but not all heterozygous combinations. These observations are in line with previous findings from the DKMS Life Science lab on HLA-DPB1 typing by NGS (unpublished data), and has implications for our subsequent chimerism analyses (see below paragraph 4.3.2).

4.3.2 NGS based HLA chimerism on serial dilutions of DNA samples

Ideally, in a heterozygous sample, 50% of the reads should be for one HLA allele of a given locus and 50% for the other. In case of mixed chimerism with 50% patient and 50% donor cells, the distribution should be 25% each for the four different HLA alleles of patient and donor. In case of HLA loss, one of the two haplotypes in the patient sample is lost while the other one is duplicated, leading to 25% reads for each of the 2 alleles in the donor and 50% reads for the remaining duplicated allele in the patient.

These distributions are different in case of haploidentical pairs, where by definition one of the two HLA alleles of all loci is shared between patient and donor, and unrelated pairs, where potentially both alleles of a given HLA locus can be mismatched. This holds true in particular for HLA-DPB1 for which matching is not a pre-requisite of donor selection (Table 4.7).

We simulated chimeric samples by performing serial dilutions of genomic DNA extracted from unrelated PBMC with mismatches at all HLA loci. NGS based HLA typing was performed on these samples using the same approach as described under 4.3.1. However, we used an about 10 times higher read-depth (5,000-10,000 reads per sample) to increase sensitivity. It should be noted that the Nextype settings for HLA type assignment were such that very low read counts (i.e. corresponding to less than 5% of the total reads) were excluded from the analysis and not indicated in the bioinformatics output. As this severely limited the potential sensitivity of the chimerism analyses, these filters were removed for the chimerism experiments. In order to determine the non-specific background of each reaction in this “unfiltered” context, we specifically looked for reads of the “patient”-specific alleles (which should be absent in the donor; Table 4.7) in 100% pure “donor” DNA. The non-specific background reads ranged from 0 to up to 200, but never exceeded 1% of the total reads. Thus, the sensitivity of our analysis varied for the different samples and within them for the different HLA alleles under analysis, but never dropped below 1%.

Table 4.7: Expected percentage of NGS read counts for HLA alleles in mismatched patient and donor pairs.

	DPB1*	100%		50%		10%	
		Patient	Donor	Classical	HLA Loss	Classical	HLA Loss
Haplo/ MUD	01:01	50	50	50	75	50	55
	02:01	50	0	25	0	5	0
	03:01	0	50	25	25	45	45
MUD	01:01	50	0	25	50	5	10
	02:01	50	0	25	0	5	0
	03:01	0	50	25	25	45	45
	04:01	0	50	25	25	45	45

Shown is as an example HLA-DPB1 with one allele mismatched and the other shared (all haploidentical and possibly unrelated pairs) or both alleles mismatched (only unrelated pairs), in 100% patient or donor sample or in mixed chimerism with 50% or 10% patient cells relative to donor cells. Shared, patient-specific and donor-specific alleles are indicated in grey, pink and green boxes, respectively.

Another important issue was the preferential amplification of one allele over the other in heterozygous samples, which is not a major problem for HLA type assignment as long as the reads exceed a certain threshold, but could be confounding factors for the presence of HLA loss variants in DNA obtained from a mixture of cells which could potentially comprise some cells with HLA loss and others without. In order to assess if preferential amplification interfered with our results on artificial chimerism curves, we compared the results obtained for the different loci, with those theoretically expected for a defined dilution. The results of this analysis are shown in Figure 4.9. In line with the data obtained for NGS based HLA typing, the observed allele counts for both alleles almost always were close to the expected results for HLA-DPB1, which has been shown not be subject of significant preferential amplification (Figure 4.9, left panel; see also paragraph 4.3.1). In contrast, the results were more variable for the loci HLA-A, B, C, DRB1, DQB1. However, it should be noted that the average chimerism obtained by combining the data of these 5 loci was always close to the expected one Figure 4.9, right panel). These data suggest that HLA-DPB1 chimerism can be assessed at the single locus level, while for the other 5 loci, combined analysis is safer because preferential amplification is an issue for some alleles. Fortunately, the only HLA locus where single mismatches in patient-donor pairs are frequent is DPB1 in MUD-HSCT, because as stated unrelated donors are very frequently (>80%) mismatched for DPB1 but are generally (>90%) matched for the remaining 5 loci. In contrast, haploidentical donors are mismatched several or all HLA loci on the unshared haplotype, and the combined data from all 6 loci will give reliable HLA chimerism results in this setting.

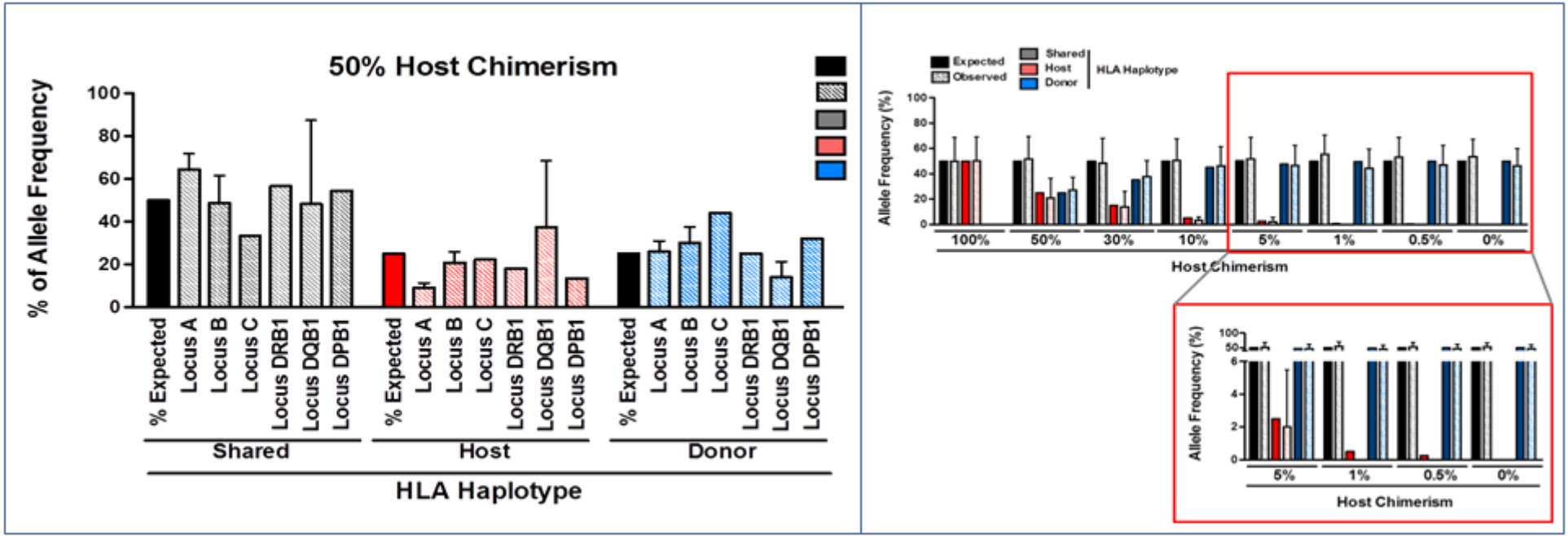


Figure 4.9: NGS-based HLA chimerism on artificial DNA mixes of two haploidentical individuals.

Shown is the percentage of reads for the shared (grey) or the unshared alleles from the host (i.e. the patient; red) and the donor (blue) at the different HLA loci. The filled bars represent the expected percentage of reads (see also Table 4.7) and the dotted bars the observed average percentage of reads. Results are shown as mean with standard error of three independent experiments. Left panel: HLA chimerism results obtained for each of the 6 individual loci at 50% chimerism. Note the low variability of results for HLA-DPB1 which are close to the expected percentages. Right panel: HLA chimerism results obtained as average of the data from the 6 individual HLA loci at different chimerism percentages. Note that the expected (and observed) percentage for the shared haplotype remains constant at 50%, while the relative percentages of the two unshared haplotypes changes with chimerism percentage. The observed overall results are in line with the expected, with a sensitivity of 0.5% (see zoomed box).

4.3.3 NGS-based detection of HLA loss relapse in a test cohort of MUD-HSCT

We applied the NGS platform for HLA chimerism to investigate HLA loss in a test cohort of 34 patients who relapsed with acute leukemia or other myeloid malignancies after MUD HSCT at the University Hospital Essen. A summary of the patient characteristics is given in Table 4.8.

Table 4.8: Summary of patients analyzed for HLA loss relapse after MUD HSCT.

	HLA Matching Status			
Method Result	**10/10 (DPmm)**	**9/10**	**8/10**	**Total**
Patients	**23**	**10**	**1**	**34**
Diagnosis				
AML	19	7	1	27
ALL	2	0	0	2
MDS/CMML/OMF	2	3	0	5
Time to Relapse	**298** (28-1514)	**414** (70-1638)	**153**	**319** (28-1638)
HLA Loss	**0**	**1**	**1**	**2**

Since a pre-requisite for performing this analysis was the presence of DNA from a blast-containing sample at relapse, we searched for such samples in the diagnostic chimerism laboratory at the Institute for Transfusion Medicine of the University Hospital Essen. We reasoned that most (99%) of post-transplantation relapses are of patient-origin [161] and are therefore associated with a mixed chimerism. DNA samples from patients who presented a mixed chimerism (at least 5% or more) by qPCR or STR for outside HLA markers in the routine diagnostic follow-up post-transplantation, and were confirmed by our colleagues at the Clinics for Bone Marrow Transplantation to have developed a hematological relapse at that time or thereafter, were selected for the study. Moreover, we restricted the diagnoses to acute leukemia or myeloid malignancies where HLA loss relapses have been described [142,145,151,158]. In order to be evaluable for HLA loss, patient and donor had to present at least one informative HLA-A, B, C, DRB1, DQB1 or DPB1 locus in the GvH direction (i.e. at least one allele present in the patient but absent in the donor).

We identified 34 samples corresponding to these criteria, all from patients who relapsed after MUD HSCT (Table 4.8). For 23 patients, the informative mismatched allele was present solely at HLA-DPB1, since they were matched with their donor for the other 5 loci (10/10; Table 4.8). In contrast, 10 and 1 patients had one and two mismatches at HLA-A, B, C, DRB1 or DQB1 and were therefore 9/10 and 8/10 matched with their donor, respectively (Table 4.9).

NGS based HLA typing was selectively performed for the informative loci, i.e. for those loci that encompassed the patient-donor HLA mismatch(es). The average read-depth for all samples was at least 5000. DNA samples from the patient pre-transplant and from the donor were analyzed in parallel to confirm typing data and, importantly, to assess the level of non-specific background reads for patient-specific alleles in 100% donor DNA (see paragraph 4.3.2). HLA chimerism was determined as the percentage of patient-specific relative to donor-specific read numbers in the relapse sample (Table 4.9). The results of HLA chimerism were then compared with those previously reported for qPCR or STR chimerism. In case of a classical relapse, HLA and qPCR/STR chimerism were expected to be congruent; in case of an HLA loss relapse, HLA chimerism is expected to be negative or at least several fold lower than qPCR /STR chimerism.

As shown in Table 4.9, in case of classical relapses, the results obtained by NGS HLA and outside HLA qPCR/STR chimerism were relatively congruent, with less than 2-fold divergence in all cases. This was different for two relapses classified as HLA loss, in which a greater than 10-fold difference between NGS HLA and outside HLA qPCR/STR chimerism was observed (UPN# 4039 and 3706, Table 4.9).

Table 4.9: NGS HLA and STR/PCR results of 34 relapses after MUD HSCT.

UPN#	HLA matching[a]	Informative HLA[b]	Chimerism (%)			
			NGS HLA[c]			
			DPB1[d]	Other HLA[d]	qPCR/STR[e]	Relapse[f]
2265	10/10	1 DPB1	7.2	NA	6.0	Classical
2713	10/10	1 DPB1	84.3	NA	75.4	Classical
3023	10/10	2 DPB1	47.0	NA	60.3	Classical
3038	10/10	1 DPB1	9.5	NA	15.3	Classical
3132	10/10	1 DPB1	11.1	NA	5.9	Classical
3135	10/10	2 DPB1	51.5	NA	39.6	Classical
3161	10/10	2 DPB1	36.5	NA	31.6	Classical
3373	10/10	1 DPB1	16.9	NA	24.3	Classical
3374	10/10	2 DPB1	18.7	NA	21.7	Classical
3582	10/10	2 DPB1	47.3	NA	74.5	Classical
3687	10/10	1 DPB1	9.7	NA	9.2	Classical
3704	10/10	1 DPB1	55.5	NA	54.5	Classical
3803	10/10	2 DPB1	25.7	NA	19.0	Classical
3838	10/10	2 DPB1	6.8	NA	13.0	Classical
3871	10/10	2 DPB1	13.0	NA	15.5	Classical
3876	10/10	1 DPB1	45.8	NA	24.5	Classical
3898	10/10	1 DPB1	65.0	NA	59.5	Classical
3970	10/10	1 DPB1	37.0	NA	17.0	Classical
3997	10/10	1 DPB1	8.5	NA	7.0	Classical
4038	10/10	2 DPB1	80.0	NA	71.0	Classical
4049	10/10	1 DPB1	8.7	NA	5.9	Classical
4087	10/10	2 DPB1	8.7	NA	12.5	Classical
4096	10/10	1 DPB1	65.5	NA	87.0	Classical
2368	9/10	2 DPB1, 1 DQB1	57.8	74.5	87.0	Classical
3032	9/10	1 DPB1, 1 B	11.1	NA[g]	7.5	Classical
3599	9/10	1 DPB1, 1 A	48.8	NA[g]	41.0	Classical
3605	9/10	2 DPB1, 1 DRB1	50.1	27.9	20.0	Classical
3667	9/10	2 DPB1, 1 A	47.1	46.4	73.9	Classical
3745	9/10	2 DPB1, 1 B	12.2	8.3	12.0	Classical
3806	9/10	1 DPB1, 1 C	43.4	60.0	25.5	Classical
3860	9/10	2 DPB1, 1 B	36.9	69.8	47.0	Classical
4039	9/10	1 DQB1[h]	NA	0.54	7.8	HLA Loss
4129	9/10	1 DPB1, 1 B	24.1	18.5	21.5	Classical
3706	8/10	1 DRB1, 1 A[h]	NA	3.5[i]	42.8	HLA Loss

[a]All donors were MUD matched for 10/10, 9/10 or 8/10 of HLA-A,B,C,DRB1,DQB1 alleles. [b]HLA locus designation of the informative allele(s) present in the patient but not in the donor, preceded by the number of patient-specific allele(s). [c]Percentage of read counts for the patient-specific allele(s) relative to the donor-specific alleles. [d]Percentage of read counts for the informative HLA-DPB1 (left column) or for another informative HLA locus (right column), if applicable. In case of 2 HLA-DPB1 mismatches, the average percentage of relative read counts for both alleles is indicated.NA; not applicable. [e]Outside HLA chimerism performed within the routine patient follow-up by qPCR or, when indicated by the suffix s, by STR. [f]Relapse was classified as classical when NGS HLA and qPCR/STR chimerism were congruent, and as HLA loss when NGS HLA was at least 10-fold lower compared to qPCR/STR chimerism. [g]NA due to incongruent typing result in the relapse sample at the informative HLA class I locus. [h]No informative HLA-DPB1 mismatch present. [i]Indicated is the percentage of read counts for the informative HLA-A allele. The percentage of read counts for the informative HLA-DRB1 allele was 40.35%.

In the 23 patients who had received a 10/10 matched MUD, all relapses were classical. In contrast, we found one definitive and another potential HLA loss relapse in 10 patients who had received MMUD. The first, UPN #3706 was a 37-year old male who had received a MMUD HSCT for AML in active disease. The MMUD was 8/10 mismatched for HLA-A and DRB1 but matched for HLA-DPB1 (Table 4.9). Despite the onset of severe aGvHD on day 30, the patient relapsed on day 153 post-transplant and succumbed 35 days later. Interestingly, divergence between outside HLA qPCR chimerism of 42.8% and NGS HLA chimerism was observed for HLA-A (3.5%) but not for HLA-DRB1 (40.3%) (Table 4.9).

This is compatible with the presence of the two mismatched alleles on two different haplotypes in this unrelated donor, with subsequent loss of the haplotype carrying the mismatched HLA-A (Table 4.10). The NGS HLA result for the mismatched HLA-A*11:01 was subsequently confirmed by qPCR chimerism using assay KMR504-A (see paragraph 4.2.2, Table 4.2), which confirmed an inside HLA chimerism of 5.7% in this sample. Unfortunately, no qPCR assay was available for DRB1, precluding confirmation of the NGS HLA chimerism result for this locus.

Table 4.10: HLA haplotype phasing in UPN #3706 and his unrelated donor.

HLA haplotype		A*	B*	C*	DRB1*	DQB1*	DPB1*
Patient	A	11:01	18:01	12:03	03:01	02:01	04:01
	B	31:01	35:03	04:01	16:02	05:02	01:01
Donor	A‘	02:01	18:01	12:03	03:01	02:01	04:01
	B‘	31:01	35:03	04:01	16:01	05:02	01:01

Haplotype A was lost in the post-transplant relapse while haplotype B was preserved, leading to selective loss of mismatched HLA-A*11:01 but not of mismatched DRB1*16:02

The second patient, UPN #4039, was a 35-year old male transplanted from a 9/10 MMUD with an isolated HLA-DQB1 mismatch (but fully matched for both HLA-DPB1 alleles) for AML M6 subtype in complete remission. The patient did not experience GvHD but relapsed 134 days after transplantation, and could be rescued by an azacytidine based salvage chemotherapy and DLI. At the date of last follow-up 417 days post-transplant (283 days post-relapse), the patient is alive and well in sustained remission. The DNA sample analyzed had an outside HLA qPCR chimerism of 7.8% compared to 0.54% by NGS (Table 4.9). Since preferential amplification was an issue for HLA-DQB1 (Figure 4.9), the possibility that this result represents an artifact cannot be ruled out. Unfortunately, no qPCR assay specific for the mismatched patient HLA-DQB1*03:01 was available to verify this point.

5. Discussion

Relapse is a major impediment to the clinical success of allo-HSCT in the cure of high risk hematologic malignancies, and the major cause of death in patients surviving 100 days after transplantation [16,44]. New advances for its prevention and treatment are therefore urgently warranted. Two major gaps to be filled to this regard are on the one hand the development of innovative tools for early detection of imminent relapse to allow timely intervention, and on the other hand the gain of new insights into the immune escape mechanisms underlying relapse after immunotherapy.

The present thesis has made a contribution to both of these issues. We showed the clinical utility of a new sensitive qPCR based chimerism approach for monitoring different relevant endpoints including relapse (Aim 1), and importantly took this method further to the detection of a specific form of “HLA loss” relapse after allo-HSCT, characterized by the genomic loss of the mismatched HLA (Aim 2). The results from these two aims of the thesis have in the meantime been published [162,163]. Both new tools have by now entered the clinical routine follow-up of transplanted patients not only at UK-Essen but also in different national and international transplant centers. We were also able to develop a cutting-edge high throughput NGS platform for the detection of HLA loss relapses in any context of HLA mismatching. Even in a so far limited test cohort of 34 patients after MUD HSCT, this method produced very interesting results, showing that HLA loss relapse is a clinical reality associated with the degree of immune pressure from HLA mismatches between patient and donor (Aim 3). Taken together, the results from this thesis thus have relevant translational implications, and set the stage for further investigations on the biology and clinical risk factors of immune escape relapse. In the following sections, I will present a critical discussion of each of the 3 parts of my thesis and a final outlook on possible future developments.

5.1 Clinical utility of qPCR for chimerism and engraftment monitoring after allo-HSCT for hematologic malignancies

When this thesis started in 2014, STR was the gold standard assay for host chimerism (HC) follow-up of transplanted patients applied by most centers including UK-Essen. This method is robust and reliable for monitoring engraftment kinetics which is the clinical endpoint chimerism analysis was initially designed for (hence the term “chimerism and engraftment monitoring”) [164]. However, STR has an intrinsically limited level of 1-5% sensitivity, due to the very low maximum amount of input DNA compatible with efficient and robust multiplex PCR which is the basis of the STR methodology. We hypothesized that added sensitivity as provided by qPCR methods targeting a single informative marker at a time would be of clinical utility first and foremost for the early detection of imminent relapse, but possibly also for a greater fine-specificity in monitoring subtle changes of HC associated with clinical events such as viral infection or donor lymphocyte infusions (DLI).

To test this hypothesis, we took advantage of a commercial assay relatively new on the market at the beginning of this thesis, based on qPCR targeting of insertion-deletion polymorphisms between patient and donor (then provided by the company Abott Molecular). We undertook a retrospective study to comparatively evaluate this qPCR method with the gold standard STR performed as part of the clinical routine follow-up, in 30 patients who had been transplanted from an unrelated donor for a hematologic malignancy at UK-Essen (Tables 4.1 and 4.2). First, we showed that several informative markers (i.e. present in the patient but absent in the donor) could be identified for all 30 patient-donor pairs, demonstrating the wide applicability of the method. Second, we found a high concordance rate of 79.6% and an overall r^2 of 0.9383 between STR and qPCR results defined as positive and negative according to two different thresholds, 1% and 0.1%, respectively. The 1-log difference in threshold was selected based on the reported sensitivity for the two assays [57,67,68], and on our own results using serial dilutions of DNA from two unrelated individuals (“artificial chimerism curves”; data not shown). Interestingly, virtually all 94 discordances were due to a negative result in STR versus a positive result in qPCR. Only 10 of these could be classified as “false positives” occurring in patients without subsequent relapse or engraftment abnormalities (Table 4.4). Interestingly, of these 10 false positive results, only one was from PB while the other nine were from BM, suggesting a very high specificity of qPCR chimerism on PB.

A possible reason for the lower specificity on BM could be the detection, by the highly sensitive qPCR, of patient-derived stromal cells present in BM but not in PB samples as previously reported [165].

The added sensitivity of qPCR over STR resulted in evident clinical utility for the detection of relapse. 8/8 informative relapses (i.e. with samples available in the 6 months before) had at least one positive result in qPCR prior to onset, compared with only 3/8 relapses in STR (Figure 4.3). Importantly, the positivity in qPCR of patients who later experienced relapse was generally sustained over several consecutive samples (Figure 4.3), while it was mostly seen on isolated BM samples by STR. It should be noted that a major limitation of this study was the lack of homogenously continuous DNA samples due to its retrospective nature. Thus, 4 additional relapses could not be evaluated because no samples were available in the months preceding relapse. A prospective study with pre-defined monitoring at regular intervals is clearly warranted to confirm our findings. This will be facilitated by the possibility to use PB which is obviously more easily accessible than BM, facilitating short-interval monitoring especially in case of suspect results. A prospective study would also be useful for dissecting the relative importance of qPCR chimerism versus the detection of tumor-specific minimal residual disease (MRD) markers for relapse prediction. The latter was precluded in this analysis by the scarceness of MRD data for the patient samples analyzed. MRD markers are generally available only for a fraction of transplanted patients [51,52], and alternative approaches to the early detection of relapse such as qPCR chimerism are welcome for these patients.

qPCR showed clinical utility also for endpoints other than relapse, in particular the fine kinetics of early engraftment. DNA samples collected in the first 7 months after transplantation were available for 21 patients who were thus informative for this endpoint. Engraftment abnormalities defined as delayed engraftment (mixed chimerism between 0.1% and 1% with subsequent drop to full donor HC) or PMC (sustained mixed chimerism above 1% without graft rejection) appeared to be influenced by the conditioning regimen, occurring in 2/2 RIC compared to 5/19 MA transplants (Tables 4.1 and 4.2), with an overall incidence of 7/21 patients (33.3%). No correlation was observed here between engraftment abnormalities and clinical outcome including aGvHD, relapse and survival. This is in contrast with a previous study of UCB HSCT in pediatric patients, where delayed low level (0.1-1%) engraftment appeared to be predictive of aGvHD [70].

This difference might be related to HLA matching status and patient age, which were both shown to be associated with the incidence of delayed engraftment in the pediatric UCB cohort and are clearly different in our adult MUD study. Interestingly, engraftment abnormalities appeared to be more frequent in patients who experienced reactivation of CMV antigenemia in the first 100 days post-transplant compared with patients who did not (3/7 [42.8%] vs 4/14 [28.5%], respectively), although the number of observations is too low to be more than observational. Of notice, in one of the patients, CMV reactivation shortly preceded the onset of PMC, with the actual time correlation between the two events demonstrated by qPCR but not by STR, which became positive only several months later (Figure 4.2). This is in line with previous reports showing an important imprinting of CMV viremia on immune reconstitution after allo-HSCT [166] [167] [168]. An additional interesting observation regards the association between early CMV reactivation and protection from AML relapse, which has been previously demonstrated by studies from UK-Essen [28,32] and also confirmed by others [29] [169]. However, recent reports have suggested that this association might be blurred by the use of ATG as GVHD prophylaxis [34] [35] [36]. This notion was not confirmed in the present cohort where the incidence of AML relapse was reduced by early CMV reactivation both in 8 transplants with ATG (0/3 relapses with CMV reactivation versus 2/5 relapses without) and in 15 transplants without (1/5 relapses with CMV reactivation versus 6/10 relapses without), although larger numbers are clearly needed to adequately address this point.

qPCR chimerism was also useful to monitor the efficacy and response kinetics of DLI for treatment of PMC or relapse. In particular, qPCR but not STR was able to provide fine-specific monitoring of the conversion from mixed to full donor chimerism after DLI for PMC or relapse, showing that this is a gradual process that proceeds over several months (Figures 4.2). This information could prove useful for clinical decision making regarding the dosage and duration of DLI, which could aid in limiting the potential toxicity of this treatment in terms of GvHD.

The data from this first part of the thesis have been published in *Ahci et al. BBMT 2017* [162]. The results have prompted a diagnostic shift from STR to qPCR as method for HC of patients transplanted at UK-Essen, a concept that is being followed by many different centers both in Germany and abroad.

5.2 A new tool for rapid and reliable diagnosis of HLA loss relapses after HSCT

Building on the positive results obtained under aim 1 (discussed in section 5.1) for qPCR chimerism using patient-specific insertion-deletion markers outside the HLA, we set out to develop a qPCR system also for markers targeting HLA mismatches between patient and donor. This second aim of the thesis was prompted by the need to develop a sensitive and user-friendly method for the diagnosis of HLA loss relapses after allo-HSCT, which are characterized by the selective genomic deletion of the HLA haplotype carrying the mismatch(es), followed by duplication of the remaining shared HLA haplotype (uniparental disomy). HLA loss was previously shown to be present in about one third of AML relapses after HSCT from haploidentical donors. Its timely diagnosis is relevant for clinical decision making, since down-tapering of immune suppression and DLI are expected to have limited efficacy against these variants that have selectively deleted the major targets of direct T cell alloreactivity. In these cases, alternative treatment strategies including the infusion of CD3+ T cell-depleted DLI enriched for innate immune cells with GvL activity such as NK cells or $\gamma\delta$ T cells, or innovative strategies such as CAR T cells or bi-specific antibodies, are preferable. Moreover, in patients fit for a second HSCT a donor mismatched for the remaining HLA haplotype should be selected in these cases, an approach which has been shown to result in a clear survival advantage [152].

The diagnosis of HLA loss was previously reserved to highly specialized research laboratories, since it was based on separate analysis and interpretation of methods with different sensitivities, i.e. chimerism assessment for outside HLA polymorphisms by STR or qPCR, and HLA typing for inside HLA polymorphisms by molecular methods such as SSOP, SSP or by SNP array analysis [145]. These methods are not only cumbersome, but require also the presence of overt relapse because of limited sensitivity, and frequently even necessitate the FACS sorting of leukemic blasts due to interference of signals from residual healthy donor hematopoiesis in the same sample. This precluded an early diagnosis of HLA loss relapse on a routine basis, although this would be of considerable clinical interest based on the above considerations.

We reasoned that these problems could be overcome through the design of qPCR assays targeted to mismatched HLA alleles, which in combination with qPCR assays for outside HLA chimerism markers could inform on the presence or absence of HLA loss in a single assay, according to the rationale outlined in Figure 4.4.

qPCR markers for HLA alleles were not commercially available, reflecting the technical challenges associated with the development of a system comprehensively targeting the highly polymorphic HLA. First, the use of insertion-deletion polymorphisms targets of outside HLA qPCR markers is not amenable to HLA, where allelic differences are almost exclusively due to SNP variability. Second, the extreme polymorphism of HLA with thousands of different reported alleles which increase in numbers at a galloping rate (Figure 1.11) makes it very difficult to develop a comprehensive system covering all possible allele combinations. Finally, for a diagnostic assay to be useful in the clinical routine, all assays have to be set at homogenous conditions. For these reasons, we focused our attention on a set of frequent alleles in worldwide populations (Table 4.5), and on HLA loci known to be frequently mismatched in HSCT not only from haploidentical but also from MUD donors, i.e. HLA-A, C and DPB1. These 3 loci have the added advantage of being slightly less polymorphic compared with HLA-B, DR and DQ, rendering the goal of designing qPCR assays more attainable. The design was performed in close collaboration with Dr. Luca Vago and Dr. Cristina Toffalori at the San Raffaele Hospital in Milan, one of the worldwide leading centers for haploidentical HSCT where HLA loss relapses were initially discovered [142]. Labor was divided into qPCR for HLA class I (A, C) and HLA class II (DPB1) to be designed at San Raffaele and UK-Essen, respectively, followed by extensive validation at both sites. The resulting publication [163] saw equal contribution co-authorship for the two first and the two last authors from both sites.

We were able to design and validate a total of 10 qPCR reactions specific for 27 HLA-A, HLA-C, and HLA-DPB1 allele groups (Table 4.5 and 4.6). All reactions worked at the same conditions (buffers, thermal cyclers) as those for outside HLA, which in the meantime had been taken over by the Dutch company GenDX who sponsored our study. They had a high efficiency of at least 90% (Table 4.6 and Figure 4.6), were highly on-target specific with few exceptions documented in our extensive validations (Figure 4.5 and 4.7), and showed a sensitivity similar to the outside markers below 0.2% (Table 4.6).

Our inside HLA assays were combined with the commercial outside HLA markers to a new kit designated HLA-KMR, suitable for the diagnosis of HLA loss relapse based on positivity for the KMR and negativity for the HLA assays (Figure 4.4). HLA-KMR was able to very reliably discriminate between classical and HLA loss relapse in a total of 20 cases of overt relapse analyzed (Figure 4.8), concordant with the results obtained previously on these samples using separate qPCR chimerism and HLA typing. Whether or not HLA-KMR is able to also predict HLA loss relapse prior to its hematological onset could not be reliably assessed since few longitudinal pre-relapse samples were available for analysis in this retrospective study. In a single informative patient, both outside and inside HLA markers were concordantly negative up to 3 months before hematological relapse univocally diagnosed as HLA loss both by HLA-KMR and by the previous separate methods (Figure 4.8).

HLA-KMR provided informative assays (i.e. positive in the patient and negative in the donor) for 70% of haploidentical HSCT and a slightly lower percentage 66% of MUD HSCT in the San Raffaele and UK-Essen series, consistent with the predicted estimates based on the frequencies of HLA alleles targeted in the kit within European populations (Table 4.6). This still leaves about one third of patients without an inside HLA marker and hence without the possibility to diagnose an HLA loss relapse by this method. The design of additional assays to cover more alleles especially at the frequently mismatched HLA-DPB1 locus could raise this frequency by 10-15% (data not shown). However, due to the technical challenges and the resulting labor-intensity of this endeavor, its cost-benefit does not appear justified especially since near to complete coverage can be obtained with the subsequently designed NGS approach for the diagnosis of HLA loss relapses (see section 5.3).

An additional potential limitation of HLA-KMR is the dependency on an internal house-keeping gene (RNAse P) for relative chimerism assessment by the $\Delta\Delta$CT method (see paragraph 3.2.3). This can be circumvented not only by NGS but also by transferring the qPCR reactions onto a digital droplet PCR (ddPCR) platform, in which qPCR is performed on the single molecule level in individual droplets and chimerism can be subsequently assessed by direct counting of positive droplets[72]. The transfer of our HLA-specific reactions onto a ddPCR platform is under way and is likely to further increase the clinical utility of the system.

HLA-KMR was developed within an international, EU-funded consortium of HSCT centers from Italy, Germany, France and Israel, with the core centers of Milan and Essen responsible for the experimental set-up and validation of the assay system, and the other centers receiving technology transfer and providing relapse samples of transplanted patients for the diagnosis of HLA loss. HLA-KMR has in the meantime been commercialized by GenDx, as part of a formal industrial cooperation with UK-Essen and San Raffaele Milan. A 2-day training course was held at UK-Essen in October 2016, coordinated by my thesis supervisor and myself, for the consortium partners from the HSCT Center UK-Dresden. HLA-KMR has entered the clinical routine in the diagnostic laboratories at UK-Essen, UK-Dresden and different other sites in Germany and abroad. Our laboratory serves as consultant for difficult cases arising within these analyses.

Taken together, the results from the second aim of my thesis have again had direct translational implications, reflected also by the industrial commercialization of our assays. The qPCR platform was deliberately chosen over more sophisticated approaches because it is amenable to most diagnostic laboratories including those from emerging countries, where allo-HSCT in particular from haploidentical donors is increasingly being implemented based on the excellent results and relatively cost-effective nature of this treatment modality [13] (Figure 1.14). These considerations are reflected by the statement from the editorial accompanying our publication of these data in Blood [163] that "…this straightforward, easily adoptable and economical tool will allow quantification of the frequency of leukemic HLA loss at the time of the main problem of haplo-HSCT for acute leukemia, which is relapse" [170].

5.3 NGS-based characterization of HLA immune escape by leukemia after immunotherapy

When this thesis started in 2014, HLA typing by NGS was being developed by specialized laboratories with very high throughput requirements, such as reference labs for unrelated HSCT donor enrolment. However, the technology was not sufficiently advanced yet to be easily implementable by laboratories without specific experience in this field. This situation changed during the first 2 years of my thesis, prompting us in the last year to embark into the set-up of an NGS platform for HLA loss diagnosis.

This decision was also promoted by the specific technical problems we had encountered in the set-up of the HLA-specific qPCR assays discussed in section 5.2, in particular the challenges associated with the design of uniform yet efficient reactions targeting extensive SNP variation in the MHC, and the possibility to provide informative assays for only roughly two thirds of patient-donor pairs. The task was facilitated by a new collaboration between our group and the DKMS Life Science lab in Dresden, one of the high-throughput HSCT donor enrolment laboratories where NGS-based HLA typing has been pioneered several years ago [159] and where extensive experience on this high-throughput typing method is being collected on thousands of donors each year.

We hypothesized that an NGS platform for HLA typing could prove useful for the diagnosis of HLA loss relapse, for the following reasons: 1) By using “generic” HLA locus specific amplification primers, any allele present in a given sample can be amplified, thereby obviating the need arising in qPCR of designing tailored specific reactions to individual HLA allele groups. 2) Chimerism assessment based on direct counting of individual reads for an informative HLA allele allows an absolute quantification and does not require comparison with an internal reference gene as in the qPCR method. 3) The high throughput nature of NGS makes this technique particularly suitable for the analysis of large sample numbers, such as required for comprehensively investigating the incidence and risk factors of HLA loss relapse in different transplant cohorts.

We first established NGS-based HLA typing by using the protocols established and published by DKMS, including the NexType software for interpretation of the results and typing alignments with the alleles reported to the HLA/IMGT database (*Lange et al. BMC 2013*). Owing to the high throughput nature of the technology, in particular for HLA typing which requires a read-depth of not more than approximately 700 to obtain reliable results, we were able to validate the technique on as many as 334 patient-donor pairs previously typed at the 2nd field level at the EFI-accredited diagnostics laboratory at the Institute for Transfusion Medicine of UK-Essen, plus 22 HLA typed reference cell lines. We found complete concordance of the NGS typings with the reported results, which had the power to almost completely resolve typing ambiguities associated with the majority of 2nd field typings obtained by conventional molecular methods.

Application of the NGS HLA typing technology to HLA chimerism required several important adjustments. First, the read-depth had to be increased by one log in order to be able to detect low levels of chimerism with a desired sensitivity of below 1%. Second and perhaps even more importantly, bioinformatical filters set for excluding low numbers of reads from the analysis in conventional HLA typings had to be removed, because the low read numbers are precisely those we are looking for in the sensitive detection of chimerism. Removal of bioinformatical filters could potentially increase the number of non-specific false positive results. However, this potential problem was solved by the concomitant analysis of 100% pure donor and patient DNA, which not only provided confirmation of HLA typing and sample identity, but also and importantly informed on the threshold number of specifically false positive reads, i.e. reads of patient-specific alleles in 100% donor DNA and vice versa. These specifically false positive read numbers were set as the individual threshold of sensitivity for each patient-donor pair, and were always below 1%. In line with this observation, the sensitivity of NGS based HLA chimerism was found in artificial chimerism curves to be below 1% (Figure 4.9). Importantly, preferential amplification of one allele over the other in heterozygous samples was not an apparent problem in the NGS analysis of HLA-DPB1, which is frequently the only informative locus in MUD HSCT [171]. For HLA-A, B, C, DRB1, DQB1 the results were more variable (Figure 4.9), however these loci are frequently informative not in an isolated manner but in conjunction with all 6 loci on an unshared HLA haplotype in haploidentical HSCT, allowing the assessment of NGS HLA chimerism on the average of the results from all 6 loci in this setting.

We were able to apply NGS HLA chimerism to a test cohort of 34 relapses after MUD HSCT at UK-Essen. One of the major problems we faced when selecting these patients was the availability of DNA samples at relapse after transplantation. The best source were DNA samples stored in the diagnostics laboratory at the Institute for Transfusion Medicine in the context of routine chimerism follow-up, where the presence of mixed chimerism was subsequently confirmed by the Clinic for Bone Marrow Transplantation to have or have not been followed by hematological relapse. Using this approach, we were able to identify a total of 104 DNA samples presenting mixed chimerism prior to relapse, which were all included for NGS HLA chimerism testing.

Unfortunately, more than two thirds of these samples yielded uninterpretable results, mainly due to the presence of an unexpectedly low read depth of less than 1000, precluding a meaningful analysis of chimerism (data not shown). The reasons for this are currently under investigation, but likely include problems regarding DNA quantification during the multiple steps of library preparation (see section 3.3). It should be noted that the entire procedure was performed manually, inevitably resulting not only in high experimental labor-intensity, but also in a certain error-proneness which might have at least partly accounted for the inconsistent read-depth. A robotic system for automated PCR set-up (EpMotion, Eppendorf, Germany) has in the meantime been acquired in the laboratory and is available for future analyses. Another problem we encountered was the specific drop-out of reads for DPB1*04:02 in some but not all samples carrying this allele (i.e. reads in the 100% positive sample not above background reads; approximate drop-out frequency of 20%, data not shown). Since DPB1*04:02 has a high allele frequency in worldwide populations (Table 4.5), it will be important to solve this problem, possibly by the design of new or additional amplification primers. In the meantime, it will be particularly important to confirm any potential HLA loss based on the missing NGS detection of patient-specific DPB1*04:02 by alternative methods such as qPCR. This will be possible in most samples due to the availability of the KMR521-DPB1 assay targeting this allele (Table 4.6). Fourteen of the 34 patient-donor pairs analyzed here carried at least one DPB1*04:02 allele, but this allele was not involved in the two relapses classified as HLA loss in this cohort.

In the 34 samples that yielded informative results, we observed at least one definitive HLA loss relapse (confirmed by allele specific qPCR) and another potential one, after 8/10 and 9/10 MMUD, respectively. In this limited cohort, the incidence of HLA loss relapse was thus 0/23 (0%) in 10/10 MUD and 1-2/10 (10-20%) in 8-9/10 MMUD. Although the number of patients analyzed is too small to draw any statistical conclusions, these findings are consistent with the notion that the risk of HLA loss relapse increases with the immunological pressure mediated by T cell alloreactivity to major HLA mismatches in unrelated HSCT. This concept has been previously supported by observations on HLA loss relapse after haploidentical HSCT, which were shown to be associated with the T cell content in the graft and the occurrence of aGvHD prior to relapse [151]. In 10/10 MUD HSCT, the major HLA mismatch is represented by HLA-DPB1, which has been shown to be target of GvL [172,173]. Therefore, the possibility that HLA-DPB1 loss might occur after 10/10 MUD HSCT

has a biological and clinical rationale. Our preliminary data suggest that this event is likely to be relatively rare. It has previously been shown that certain HLA-DPB1 mismatch combinations give rise to stronger T cell alloreactivity and are hence clinically less well tolerated than others, leading to the classification of HLA-DPB1 mismatches into so-called “non-permissive” and “permissive”, respectively [174]. This raises the interesting possibility that the stronger immunological pressure from non-permissive compared to permissive HLA-DPB1 mismatches might translate into a higher frequency of HLA loss mismatches in the former than in the latter. Thirty-two patient-donor pairs in our cohort had HLA-DPB1 mismatches in GvH direction and 7 (21.8%) of these were non-permissive, which is in line with the HLA-DPB1 allele frequencies reported in the European population. Larger studies are clearly warranted to establish the incidence of HLA loss relapse in 10/10 MUD HSCT, and the potential role of non-permissive mismatches in this context.

The finding of certainly one and potentially two HLA loss relapses even in the small cohort of only 10 MMUD transplants analyzed demonstrates that this type of relapse is a clinical reality also in settings other than haploidentical transplantation, making a case for routine diagnostics of HLA loss in all MMUD relapses. The HLA-KMR approach developed by us will prove useful for this purpose. Of note, we observed the selective loss of the mismatched HLA class I A allele but not of the second mismatched HLA class II DRB1 allele in the patient with HLA loss relapse after 8/10 MMUD (Table 4.9), with HLA-A loss subsequently confirmed by one of our specific HLA-KMR qPCR assays. This suggests LOH for the haplotype carrying the retained mismatched HLA-DRB1 after deletion of the haplotype carrying the mismatched HLA-A. It should be noted that we did not find evidence for uniparental disomy, i.e. for duplication of the haplotype carrying the HLA-DRB1 mismatch, since the NGS chimerism on DRB1 was not different from the qPCR chimerism outside HLA (both approximately 40%; Table 4.9). This suggests that either this was a case of simple LOH without uniparental disomy, or alternatively that the accuracy of NGS-based copy number assessment for HLA-DRB1 might be too limited to detect this condition, for instance due to problems associated with preferential amplification at this locus. The finding that in the presence of a combined mismatch for HLA class I and II, greater immunological pressure was evidently mediated by HLA class I than by HLA class II is of potential interest and in line with reports from solid tumors where HLA class I LOH and somatic mutations were shown to occur in relapses after chemotherapy [129,175].

The second potential case of HLA loss involved an isolated HLA-DQB1 mismatch. It should be noted that this sample had a relatively low level of chimerism of only 7.8%, and preferential amplification was an issue for HLA-DQB1 (Table 4.9). Therefore, the possibility that this HLA loss represents an artifact cannot be completely ruled out, and confirmation of HLA loss by the design of a qPCR assay for the mismatched HLA-DQB1*03:01 is needed to clarify this point. Clinically, this patient could be successfully treated by chemotherapy and DLI. The apparent efficacy of DLI in treating an HLA loss relapse would be somewhat surprising, since the major target of T cell alloreactivity would be lost by the relapse undergoing this specific immune escape. However, minor histocompatibility antigens might have been residual targets of GvL in this case.

Taken together, the validation and first test application of an innovative NGS platform for HLA chimerism and HLA loss relapse detection yielded interesting results. However it still requires optimization by robotics, and the issue of specific allele drop-outs and preferential amplification at individual loci has to be dealt with. Once these issues are solved, we plan to proceed with the analysis of the entire set of 108 relapse samples from UK-Essen, as well as over 100 additional relapses sent to us from national collaborators at UK-Dresden, UK-Hamburg and UK-Düsseldorf.

5.4 Outlook on future developments

Apart from providing a qPCR based platform for sensitive detection of post-transplant relapse in general and HLA loss relapse in particular, the results from this thesis allowed us to obtain a first glimpse into the incidence and risk factors of the latter form of relapse after allo-HSCT from unrelated donors. This sets the stage for future investigations. First, the NGS platform for HLA chimerism we developed will allow us to perform a large scale analysis of HLA loss relapse in different transplant contexts, including haploidentical, MUD, MMUD and UCB HSCT. In a national and international effort involving over 30 centers from all over the world, we were able to collect more than 600 relapse samples to be analyzed in the two collaborating centers UK-Essen (2-300 samples from national collaborators in Germany) and San Raffaele Milan (the remaining samples from international collaborators). This represents the largest post-transplantation relapse cohort ever to be analyzed for this specific form of HLA immune escape.

Second and importantly, the NGS HLA chimerism approach not only provides information on relative HLA allele copy numbers for the detection of genomic HLA loss, but also on the nucleotide sequence of individual alleles. This potentially enables the detection of somatic neo-mutations targeted to HLA as a new, additional form of immune escape. Non-silent HLA mutations were recently reported to be present in 266/7930 (3.4%) of mainly solid tumors not subjected to pressure by allogeneic immune therapy [129]. It is possible and even likely that this frequency might be even higher in relapses of hematologic malignancies after allo-HSCT, i.e. tumor cells managing to evade the immune attack from allogeneic donor cells. The pipeline used for the identification of somatic HLA neo-mutations in the previous study regarded only HLA class I, and was based on a whole exome sequencing approach from which HLA data were extrapolated [129].

Our strategy of targeted sequencing allows for the analysis of both HLA class I and HLA class II, which are both potentially relevant targets of immune escape for hematological malignancies which, in contrast to solid tumors, frequently express both HLA classes. For these analyses, it will be important to include not only exons 2 and 3, which are sufficient for the detection of genomic HLA loss, but also exons beyond that region, i.e. exons 1-7 for HLA class I and exons 1-5 for HLA class II (Figure 1.7), since frequent HLA neo-mutations were shown to affect exon 4, outside of the classical exon 2-3 region encoding the most of the HLA variability [129]. The set-up of the relevant PCR system is likely to pose an inferior challenge to this regard than the bioinformatical platform required for a meaningful analysis, which will be set in collaboration with the biostatistics team of the University Duisburg Essen. The possibility to investigate somatic neo-mutations in HLA will be of interest for relapses not only of hematologic malignancies after allo-HSCT, but also for hematologic and non-hematologic tumors after immunotherapy with new therapeutic agents such as CAR T cells, bi-specific antibodies and checkpoint inhibitors. The future developments of this project are therefore expected to reach out to different areas of immunotherapy beyond allo-HSCT, thereby opening potential new opportunities of national and international networking.

6. Summary

Relapse of malignant disease is the most frequent cause of treatment failure in allogeneic hematopoietic stem cell transplantation (allo-HSCT), often due to selective genomic loss of the mismatched human leukocyte antigens (“HLA loss”). The diagnosis of this condition is relevant for adopting appropriate treatment strategies and assessing its relevance in different transplant settings, but is precluded by technical challenges associated with the extreme polymorphism in HLA. The aim of the present thesis was to close this gap by 1) investigating the clinical utility of quantitative PCR (qPCR) for chimerism monitoring after HSCT; 2) developing HLA-specific qPCR assays to detect HLA loss relapse; and 3) establishing new next generation sequencing (NGS) protocols for the high throughput detection of HLA loss relapse.

In the first aim, published in *Ahci et al., Biol Blood Marrow Transplant 2017*, we retrospectively tested chimerism after 30 allo-HSCT at UK-Essen, in parallel by standard short tandem repeat (STR) and a new commercial qPCR method targeting insertion-deletion polymorphisms outside HLA. We demonstrated a 1-log higher sensitivity of qPCR, resulting in clinical utility for early relapse detection and other clinical endpoints including engraftment. These results led to a diagnostic shift from STR to qPCR for the follow-up of allo-HSCT at UK-Essen. In the second aim, published in *Ahci & Toffalori et al., Blood 2017*, we developed qPCR chimerism for single nucleotide polymorphisms within the HLA system, providing a user-friendly, robust and sensitive method for the detection of HLA loss relapse which combines chimerism outside and inside HLA in a single “HLA-KMR” system under industrial sponsorship and commercialization. In the third aim, we developed NGS-based HLA typing in collaboration with the DKMS Life Science Lab leader in the field, adapted this approach to the detection of HLA chimerism and applied it to 34 relapses after unrelated donor HSCT at UK-Essen. This allowed us to identify at least one HLA loss relapse occurring after partially HLA mismatched HSCT, further substantiating the clinical relevance of HLA loss as mechanism underlying relapse. Taken together, the data from my thesis set the stage for further analyses regarding the incidence and risk factors of HLA loss relapse in different transplant contexts through national and international collaborations, and the investigation of somatic neo-mutations in HLA as a new mechanism of immune escape after cancer immunotherapy.

7. Zusammenfassung

Die häufigste Ursache des Therapieversagens der allogenen hämatopoetischen Stammzelltransplantation (allo-HSZT) zur Behandlung bösartiger Bluterkrankungen sind Krankheitsrückfälle, welch häufig durch den genomischen Verlust der patienten-spezifischen HLA Gewebeantigene gekennzeichnet sind („HLA Verlust“). Die Diagnose dieses gezielten Immunevasionsmechanismus ist aufgrund der großen Variabilität des HLA Systems mit erheblichen technischen Schwierigkeiten verbunden, obwohl sie von großer klinischer und wissenschaftlicher Bedeutung wäre. Das Ziel der vorliegenden Doktorarbeit war es, diese Lücke zu schließen und durch 1) Untersuchung des klinischen Vorteils der Chimärismusbestimmung nach HSZT mittels quantitativer PCR (qPCR); 2) Entwicklung von HLA-spezifischen qPCR Reaktionen zur Erkennung des HLA Verlust Rückfalls; 3) Aufbau von next generation sequencing (NGS) zur Hochdurchsatzbestimmung von HLA Verlust Rezidiven.

Für das erste Ziel, veröffentlicht in *Ahci et al., Biol Blood Marrow Transplant 2017*, wurden 30 allo-HSZT vom UK-Essen vergleichend mit der Standardmethode short tandem repeat (STR) und einem neuen kommerziellen qPCR Ansatz auf Chimärismus getestet. Die höhere Sensitivität der qPCR war klinisch für die Beurteilung von Rezidiven und anderen klinischen Endpunkten von Vorteil und wird seither routinemäßig am UK-Essen eingesetzt. Für das zweite Ziel, veröffentlicht in *Ahci & Toffalori et al., Blood 2017*, wurden qPCR Reaktionen für eine Anzahl von HLA Allelen entwickelt um eine nutzerfreundliche, robuste und sensitive Methode zur Erkennung von HLA Verlust Rezidiven zu erstellen, welche als „HLA-KMR“ industrielle Verwertung gefunden hat. Für das dritte Ziel wurde eine NGS Plattform zur HLA Typisierung in Kooperation mit dem DKMS Life Science Lab entwickelt, auf den HLA Chimärismus adaptiert und anhand von 34 Rückfallproben von am UK-Essen transplantierten Patienten getestet. Selbst in dieser limitierten Kohorte wurde mindestens ein HLA Verlust Rezidiv nach partiell HLA differenter HSZT entdeckt, was die klinische Relevanz dieses Immunevasionsmechanismus unterstreicht. Die in dieser Doktorarbeit erzielten Resultate werden nun die Untersuchung der Inzidenz und Risikofaktoren von HLA Verlustrezidiven nach allo-HSZT in großen nationalen und internationalen Studien ermöglichen. Zudem wird die NGS Plattform zur Analyse von somatischen HLA Neomutationen als potentiellem neuen Immunevasions-mechanismus nach Tumorimmuntherapie genutzt werden.

8. References

1. Thomas ED, Lochte HL, Jr., Lu WC, Ferrebee JW. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med*. 1957;257(11):491-496.

2. Appelbaum FR. Hematopoietic-cell transplantation at 50. *N Engl J Med*. 2007;357(15):1472-1475.

3. Thomas ED, Storb R, Clift RA, et al. Bone-marrow transplantation (second of two parts). *N Engl J Med*. 1975;292(17):895-902.

4. Thomas E, Storb R, Clift RA, et al. Bone-marrow transplantation (first of two parts). *N Engl J Med*. 1975;292(16):832-843.

5. Weiden PL, Storb R, Tsoi MS, Graham TC, Lerner KG, Thomas ED. Infusion of donor lymphocytes into stable canine radiation chimeras: implications for mechanism of transplantation tolerance. *J Immunol*. 1976;116(5):1212-1219.

6. Weiden PL, Flournoy N, Thomas ED, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. *N Engl J Med*. 1979;300(19):1068-1073.

7. Thomas ED, Buckner CD, Sanders JE, et al. Marrow transplantation for thalassaemia. *Lancet*. 1982;2(8292):227-229.

8. Johnson FL, Look AT, Gockerman J, Ruggiero MR, Dalla-Pozza L, Billings FT, 3rd. Bone-marrow transplantation in a patient with sickle-cell anemia. *N Engl J Med*. 1984;311(12):780-783.

9. Sheridan WP, Begley CG, Juttner CA, et al. Effect of peripheral-blood progenitor cells mobilised by filgrastim (G-CSF) on platelet recovery after high-dose chemotherapy. *Lancet*. 1992;339(8794):640-644.

10. Weaver CH, Hazelton B, Birch R, et al. An analysis of engraftment kinetics as a function of the CD34 content of peripheral blood progenitor cell collections in 692 patients after the administration of myeloablative chemotherapy. *Blood*. 1995;86(10):3961-3969.

11. Welte K, Gabrilove J, Bronchud MH, Platzer E, Morstyn G. Filgrastim (r-metHuG-CSF): the first 10 years. *Blood*. 1996;88(6):1907-1929.

12. Aversa F, Tabilio A, Velardi A, et al. Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N Engl J Med*. 1998;339(17):1186-1193.

13. Passweg JR, Baldomero H, Bader P, et al. Use of haploidentical stem cell transplantation continues to increase: the 2015 European Society for Blood and Marrow Transplant activity survey report. *Bone Marrow Transplant*. 2017;52(6):811-817.

14. Majhail NS, Chitphakdithai P, Logan B, et al. Significant improvement in survival after unrelated donor hematopoietic cell transplantation in the recent era. *Biol Blood Marrow Transplant*. 2015;21(1):142-150.

15. Gooley TA, Chien JW, Pergam SA, et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. *N Engl J Med*. 2010;363(22):2091-2101.

16. Scott EC, Hari P, Sharma M, et al. Post-Transplant Outcomes in High-Risk Compared with Non-High-Risk Multiple Myeloma: A CIBMTR Analysis. *Biol Blood Marrow Transplant*. 2016;22(10):1893-1899.

17. Gibson CJ, Lindsley RC, Tchekmedyian V, et al. Clonal Hematopoiesis Associated With Adverse Outcomes After Autologous Stem-Cell Transplantation for Lymphoma. *J Clin Oncol*. 2017;35(14):1598-1605.

18. Artz AS. From biology to clinical practice: aging and hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2012;18(1 Suppl):S40-45.

19. Lim Z, Brand R, Martino R, et al. Allogeneic hematopoietic stem-cell transplantation for patients 50 years or older with myelodysplastic syndromes or secondary acute myeloid leukemia. *J Clin Oncol*. 2010;28(3):405-411.

20. Storb R. Reduced-intensity conditioning transplantation in myeloid malignancies. *Curr Opin Oncol*. 2009;21 Suppl 1:S3-5.

21. Smith TJ, Khatcheressian J, Lyman GH, et al. 2006 update of recommendations for the use of white blood cell growth factors: an evidence-based clinical practice guideline. *J Clin Oncol*. 2006;24(19):3187-3205.

22. Gluckman E, Rocha V. Cord blood transplantation: state of the art. *Haematologica*. 2009;94(4):451-454.

23. Storek J. Immunological reconstitution after hematopoietic cell transplantation - its relation to the contents of the graft. *Expert Opin Biol Ther*. 2008;8(5):583-597.

24. Boeckh M, Geballe AP. Cytomegalovirus: pathogen, paradigm, and puzzle. *J Clin Invest*. 2011;121(5):1673-1680.

25. Schmidt-Hieber M, Labopin M, Beelen D, et al. CMV serostatus still has an important prognostic impact in de novo acute leukemia patients after allogeneic stem cell transplantation: a report from the Acute Leukemia Working Party of EBMT. *Blood*. 2013;122(19):3359-3364.

26. Schmidt-Hieber M, Schwarck S, Stroux A, et al. Immune reconstitution and cytomegalovirus infection after allogeneic stem cell transplantation: the important impact of in vivo T cell depletion. *Int J Hematol*. 2010;91(5):877-885.

27. Teira P, Battiwalla M, Ramanathan M, et al. Early cytomegalovirus reactivation remains associated with increased transplant-related mortality in the current era: a CIBMTR analysis. *Blood*. 2016;127(20):2427-2438.

28. Elmaagacli AH, Steckel NK, Koldehoff M, et al. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood*. 2011;118(5):1402-1412.

29. Green ML, Leisenring WM, Xie H, et al. CMV reactivation after allogeneic HCT and relapse risk: evidence for early protection in acute myeloid leukemia. *Blood*. 2013;122(7):1316-1324.

30. Guenounou S, Borel C, Berard E, et al. Prognostic impact of viral reactivations in acute myeloid leukemia patients undergoing allogeneic stem cell transplantation in first complete response. *Medicine (Baltimore)*. 2016;95(48):e5356.

31. Jang JE, Kim SJ, Cheong JW, et al. Early CMV replication and subsequent chronic GVHD have a significant anti-leukemic effect after allogeneic HSCT in acute myeloid leukemia. *Ann Hematol*. 2015;94(2):275-282.

32. Koldehoff M, Ross SR, Duhrsen U, Beelen DW, Elmaagacli AH. Early CMV-replication after allogeneic stem cell transplantation is associated with a reduced relapse risk in lymphoma. *Leuk Lymphoma*. 2017;58(4):822-833.

33. Takenaka K, Nishida T, Asano-Mori Y, et al. Cytomegalovirus Reactivation after Allogeneic Hematopoietic Stem Cell Transplantation is Associated with a Reduced Risk of Relapse in Patients with Acute Myeloid Leukemia Who Survived to Day 100 after Transplantation: The Japan Society for Hematopoietic Cell Transplantation Transplantation-related Complication Working Group. *Biol Blood Marrow Transplant*. 2015;21(11):2008-2016.

34. Bao X, Zhu Q, Xue S, et al. Cytomegalovirus induces strong antileukemic effect in acute myeloid leukemia patients following sibling HSCT without ATG-containing regimen. *Am J Transl Res*. 2016;8(2):653-661.

35. Busca A, Passera R, Pini M, et al. The use of ATG abrogates the antileukemic effect of cytomegalovirus reactivation in patients with acute myeloid leukemia receiving grafts from unrelated donors. *Am J Hematol*. 2015;90(6):E117-121.

36. Manjappa S, Bhamidipati PK, Stokerl-Goldstein KE, et al. Protective effect of cytomegalovirus reactivation on relapse after allogeneic hematopoietic cell transplantation in acute myeloid leukemia patients is influenced by conditioning regimen. *Biol Blood Marrow Transplant*. 2014;20(1):46-52.

37. van Bekkum DW. Bone marrow transplantation. *Transplant Proc*. 1977;9(1):147-154.

38. van Bekkum DW, Knaan S. Role of bacterial microflora in development of intestinal lesions from graft-versus-host reaction. *J Natl Cancer Inst*. 1977;58(3):787-790.

39. Staffas A, Burgos da Silva M, van den Brink MR. The intestinal microbiota in allogeneic hematopoietic cell transplant and graft-versus-host disease. *Blood*. 2017;129(8):927-933.

40. Zeiser R, Blazar BR. Pathophysiology of Chronic Graft-versus-Host Disease and Therapeutic Targets. *N Engl J Med*. 2017;377(26):2565-2579.

41. Zeiser R, Blazar BR. Acute Graft-versus-Host Disease - Biologic Process, Prevention, and Therapy. *N Engl J Med*. 2017;377(22):2167-2179.

42. Bruserud O, Tvedt TH, Paulsen PQ, et al. Extracorporeal photopheresis (photochemotherapy) in the treatment of acute and chronic graft versus host disease: immunological mechanisms and the results from clinical studies. *Cancer Immunol Immunother*. 2014;63(8):757-777.

43. Jagasia M, Zeiser R, Arbushites M, Delaite P, Gadbaw B, Bubnoff NV. Ruxolitinib for the treatment of patients with steroid-refractory GVHD: an introduction to the REACH trials. *Immunotherapy*. 2018;10(5):391-402.

44. Soiffer RJ, Chen YB. Pharmacologic agents to prevent and treat relapse after allogeneic hematopoietic cell transplantation. *Hematology Am Soc Hematol Educ Program*. 2017;2017(1):699-707.

45. Greinix HT, Loddenkemper C, Pavletic SZ, et al. Diagnosis and staging of chronic graft-versus-host disease in the clinical practice. *Biol Blood Marrow Transplant*. 2011;17(2):167-175.

46. Warren EH, Deeg HJ. Dissecting graft-versus-leukemia from graft-versus-host-disease using novel strategies. *Tissue Antigens*. 2013;81(4):183-193.

47. de Witte MA, Kuball J, Miller JS. NK Cells and gammadeltaT Cells for Relapse Protection After Allogeneic Hematopoietic Cell Transplantation (HCT). *Curr Stem Cell Rep*. 2017;3(4):301-311.

48. Roddie C, Peggs KS. Donor lymphocyte infusion following allogeneic hematopoietic stem cell transplantation. *Expert Opin Biol Ther*. 2011;11(4):473-487.

49. Peggs KS, Mackinnon S. Cytomegalovirus: the role of CMV post-haematopoietic stem cell transplantation. *Int J Biochem Cell Biol*. 2004;36(4):695-701.

50. Andreani M, Testi M, Battarra M, et al. Relationship between mixed chimerism and rejection after bone marrow transplantation in thalassaemia. *Blood Transfus*. 2008;6(3):143-149.

51. Ommen HB. Monitoring minimal residual disease in acute myeloid leukaemia: a review of the current evolving strategies. *Ther Adv Hematol*. 2016;7(1):3-16.

52. Thiede C, Bornhauser M, Ehninger G. Strategies and clinical implications of chimerism diagnostics after allogeneic hematopoietic stem cell transplantation. *Acta Haematol*. 2004;112(1-2):16-23.

53. Kristt D, Stein J, Yaniv I, Klein T. Assessing quantitative chimerism longitudinally: technical considerations, clinical applications and routine feasibility. *Bone Marrow Transplant*. 2007;39(5):255-268.

54. Lawler M, Humphries P, McCann SR. Evaluation of mixed chimerism by in vitro amplification of dinucleotide repeat sequences using the polymerase chain reaction. *Blood*. 1991;77(11):2504-2514.

55. McCann SR, Crampe M, Molloy K, Lawler M. Hemopoietic chimerism following stem cell transplantation. *Transfus Apher Sci*. 2005;32(1):55-61.

56. Elmaagacli AH, Runkel K, Steckel N, et al. A comparison of chimerism and minimal residual disease between four different allogeneic transplantation methods in patients with chronic myelogenous leukemia in first chronic phase. *Bone Marrow Transplant*. 2001;27(8):809-815.

57. Frankfurt O, Zitzner JR, Tambur AR. Real-time qPCR for chimerism assessment in allogeneic hematopoietic stem cell transplants from unrelated adult and double umbilical cord blood. *Hum Immunol*. 2015;76(2-3):155-160.

58. Gardiner N, Lawler M, O'Riordan J, DeArce M, Humphries P, McCann SR. Persistent donor chimaerism is consistent with disease-free survival following BMT for chronic myeloid leukaemia. *Bone Marrow Transplant*. 1997;20(3):235-241.

59. Masmas TN, Madsen HO, Petersen SL, et al. Evaluation and automation of hematopoietic chimerism analysis based on real-time quantitative polymerase chain reaction. *Biol Blood Marrow Transplant*. 2005;11(7):558-566.

60. Alizadeh M, Bernard M, Danic B, et al. Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. *Blood*. 2002;99(12):4618-4625.

61. Jimenez-Velasco A, Barrios M, Roman-Gomez J, et al. Reliable quantification of hematopoietic chimerism after allogeneic transplantation for acute leukemia using amplification by real-time PCR of null alleles and insertion/deletion polymorphisms. *Leukemia*. 2005;19(3):336-343.

62. Maas F, Schaap N, Kolen S, et al. Quantification of donor and recipient hemopoietic cells by real-time PCR of single nucleotide polymorphisms. *Leukemia*. 2003;17(3):621-629.

63. Bach C, Tomova E, Goldmann K, et al. Monitoring of hematopoietic chimerism by real-time quantitative PCR of micro insertions/deletions in samples with low DNA quantities. *Transfus Med Hemother*. 2015;42(1):38-45.

64. Kim SY, Jeong MH, Park N, et al. Chimerism monitoring after allogeneic hematopoietic stem cell transplantation using quantitative real-time PCR of biallelic insertion/deletion polymorphisms. *J Mol Diagn*. 2014;16(6):679-688.

65. Kletzel M, Huang W, Olszewski M, Khan S. Validation of chimerism in pediatric recipients of allogeneic hematopoietic stem cell transplantation (HSCT) a comparison between two methods: real-time PCR (qPCR) vs. variable number tandem repeats PCR (VNTR PCR). *Chimerism*. 2013;4(1):1-8.

66. Koldehoff M, Steckel NK, Hlinka M, Beelen DW, Elmaagacli AH. Quantitative analysis of chimerism after allogeneic stem cell transplantation by real-time polymerase chain reaction with single nucleotide polymorphisms, standard tandem repeats, and Y-chromosome-specific sequences. *Am J Hematol*. 2006;81(10):735-746.

67. Jacque N, Nguyen S, Golmard JL, et al. Chimerism analysis in peripheral blood using indel quantitative real-time PCR is a useful tool to predict post-transplant relapse in acute leukemia. *Bone Marrow Transplant*. 2015;50(2):259-265.

68. Qin XY, Li GX, Qin YZ, et al. Quantitative chimerism: an independent acute leukemia prognosis indicator following allogeneic hematopoietic SCT. *Bone Marrow Transplant*. 2014;49(10):1269-1277.

69. Willasch AM, Kreyenberg H, Shayegi N, et al. Monitoring of hematopoietic chimerism after transplantation for pediatric myelodysplastic syndrome: real-time or conventional short tandem repeat PCR in peripheral blood or bone marrow? *Biol Blood Marrow Transplant*. 2014;20(12):1918-1925.

70. Elkaim E, Picard C, Galambrun C, et al. Peripheral blood cells chimerism after unrelated cord blood transplantation in children: kinetics, predictive factors and impact on post-transplant outcome. *Br J Haematol*. 2014;166(4):557-565.

71. George D, Czech J, John B, Yu M, Jennings LJ. Detection and quantification of chimerism by droplet digital PCR. *Chimerism*. 2013;4(3):102-108.

72. Hindson BJ, Ness KD, Masquelier DA, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal Chem*. 2011;83(22):8604-8610.

73. Weaver S, Dube S, Mir A, et al. Taking qPCR to a higher level: Analysis of CNV reveals the power of high throughput qPCR to enhance quantitative resolution. *Methods*. 2010;50(4):271-276.

74. Aloisio M, Licastro D, Caenazzo L, et al. A technical application of quantitative next generation sequencing for chimerism evaluation. *Mol Med Rep*. 2016;14(4):2967-2974.

75. Karl L. Zur Kenntnis der antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe. *Zentralblatt Bakteriologie* 1900;27:357–362.

76. Dausset J. [Iso-leuko-antibodies]. *Acta Haematol*. 1958;20(1-4):156-166.

77. Payne R, Rolfs MR. Fetomaternal leukocyte incompatibility. *J Clin Invest*. 1958;37(12):1756-1763.

78. Van Rood JJ, Eernisse JG, Van Leeuwen A. Leucocyte antibodies in sera from pregnant women. *Nature*. 1958;181(4625):1735-1736.

79. Thorsby E. A short history of HLA. *Tissue Antigens*. 2009;74(2):101-116.

80. Thorsby E. On the future of HLA. *Tissue Antigens*. 2011;78(4):229-240.

81. Stiehm ER. Joseph A. Bellanti (ed) immunology IV: clinical applications in health and disease. *J Clin Immunol*. 2012;32(3):647.

82. Bodmer WF, Trowsdale J, Young J, Bodmer J. Gene clusters and the evolution of the major histocompatibility system. *Philos Trans R Soc Lond B Biol Sci*. 1986;312(1154):303-315.

83. Goldberg AC, Rizzo LV. MHC structure and function - antigen presentation. Part 1. *Einstein (Sao Paulo)*. 2015;13(1):153-156.

84. Goldberg AC, Rizzo LV. MHC structure and function - antigen presentation. Part 2. *Einstein (Sao Paulo)*. 2015;13(1):157-162.

85. Horton R, Wilming L, Rand V, et al. Gene map of the extended human MHC. *Nat Rev Genet*. 2004;5(12):889-899.

86. Robinson J, Halliwell JA, Hayhurst JD, Flicek P, Parham P, Marsh SG. The IPD and IMGT/HLA database: allele variant databases. *Nucleic Acids Res*. 2015;43(Database issue):D423-431.

87. McDevitt H. The discovery of linkage between the MHC and genetic control of the immune response. *Immunol Rev*. 2002;185:78-85.

88. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature*. 1987;329(6139):506-512.

89. Ghosh P, Amaya M, Mellins E, Wiley DC. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature*. 1995;378(6556):457-462.

90. Neefjes J, Jongsma ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol*. 2011;11(12):823-836.

91. Prevosto C, Usmani MF, McDonald S, et al. Allele-Independent Turnover of Human Leukocyte Antigen (HLA) Class Ia Molecules. *PLoS One*. 2016;11(8):e0161011.

92. Reith W, LeibundGut-Landmann S, Waldburger JM. Regulation of MHC class II gene expression by the class II transactivator. *Nat Rev Immunol*. 2005;5(10):793-806.

93. Hulsdunker J, Ottmuller KJ, Neeff HP, et al. Neutrophils provide cellular communication between ileum and mesenteric lymph nodes at graft-versus-host disease onset. *Blood*. 2018.

94. Hershberg RM, Cho DH, Youakim A, et al. Highly polarized HLA class II antigen processing and presentation by human intestinal epithelial cells. *J Clin Invest*. 1998;102(4):792-803.

95. Steimle V, Siegrist CA, Mottet A, Lisowska-Grospierre B, Mach B. Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science*. 1994;265(5168):106-109.

96. Pos W, Sethi DK, Wucherpfennig KW. Mechanisms of peptide repertoire selection by HLA-DM. *Trends Immunol*. 2013;34(10):495-501.

97. Zinkernagel RM, Doherty PC. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature*. 1974;248(5450):701-702.

98. Spierings E. Minor histocompatibility antigens: past, present, and future. *Tissue Antigens*. 2014;84(4):374-360.

99. Geneugelijk K, Spierings E. Matching donor and recipient based on predicted indirectly recognizable human leucocyte antigen epitopes. *Int J Immunogenet*. 2018;45(2):41-53.

100. Felix NJ, Allen PM. Specificity of T-cell alloreactivity. *Nat Rev Immunol*. 2007;7(12):942-953.

101. Robinson J, Halliwell JA, McWilliam H, Lopez R, Parham P, Marsh SG. The IMGT/HLA database. *Nucleic Acids Res*. 2013;41(Database issue):D1222-1227.

102. Bentley G, Higuchi R, Hoglund B, et al. High-resolution, high-throughput HLA genotyping by next-generation sequencing. *Tissue Antigens*. 2009;74(5):393-403.

103. Lind C, Ferriola D, Mackiewicz K, et al. Next-generation sequencing: the solution for high-resolution, unambiguous human leukocyte antigen typing. *Hum Immunol*. 2010;71(10):1033-1042.

104. A. GP. The antigenic basis of tumour transplantation. *The Journal of Pathology and Bacteriology* May 1937;Volume 44(Issue 3):691-697.

105. Medawar PB. A second study of the behaviour and fate of skin homografts in rabbits: A Report to the War Wounds Committee of the Medical Research Council. *J Anat*. 1945;79(Pt 4):157-176 154.

106. Gorer PA. The significance of studies with transplanted tumours. *Br J Cancer*. 1948;2(2):103-107.

107. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*. 1990;75(3):555-562.

108. Hansen JA, Clift RA, Thomas ED, Buckner CD, Storb R, Giblett ER. Transplantation of marrow from an unrelated donor to a patient with acute leukemia. *N Engl J Med*. 1980;303(10):565-567.

109. Lee SJ, Klein J, Haagenson M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood*. 2007;110(13):4576-4583.

110. Gragert L, Eapen M, Williams E, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *N Engl J Med*. 2014;371(4):339-348.

111. Luznik L, O'Donnell PV, Symons HJ, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. 2008;14(6):641-650.

112. Bashey A, Zhang X, Sizemore CA, et al. T-cell-replete HLA-haploidentical hematopoietic transplantation for hematologic malignancies using post-transplantation cyclophosphamide results in outcomes equivalent to those of contemporaneous HLA-matched related and unrelated donor transplantation. *J Clin Oncol*. 2013;31(10):1310-1316.

113. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375-2390.

114. Taga T, Tomizawa D, Takahashi H, Adachi S. Acute myeloid leukemia in children: Current status and future directions. *Pediatr Int*. 2016;58(2):71-80.

115. Joo WD, Visintin I, Mor G. Targeted cancer therapy--are the days of systemic chemotherapy numbered? *Maturitas*. 2013;76(4):308-314.

116. Stelljes M, Krug U, Beelen DW, et al. Allogeneic transplantation versus chemotherapy as postremission therapy for acute myeloid leukemia: a prospective matched pairs analysis. *J Clin Oncol*. 2014;32(4):288-296.

117. Bonini C, Ferrari G, Verzeletti S, et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science*. 1997;276(5319):1719-1724.

118. Bejanyan N, Weisdorf DJ, Logan BR, et al. Survival of patients with acute myeloid leukemia relapsing after allogeneic hematopoietic cell transplantation: a center for international blood and marrow transplant research study. *Biol Blood Marrow Transplant*. 2015;21(3):454-459.

119. Bonini C, Peccatori J, Stanghellini MT, et al. Haploidentical HSCT: a 15-year experience at San Raffaele. *Bone Marrow Transplant*. 2015;50 Suppl 2:S67-71.

120. Mavilio F, Ferrari G, Rossini S, et al. Peripheral blood lymphocytes as target cells of retroviral vector-mediated gene transfer. *Blood*. 1994;83(7):1988-1997.

121. Kelly TK, De Carvalho DD, Jones PA. Epigenetic modifications as therapeutic targets. *Nat Biotechnol*. 2010;28(10):1069-1078.

122. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis*. 2010;31(1):27-36.

123. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 2001;344(14):1031-1037.

124. Druker BJ, Guilhot F, O'Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*. 2006;355(23):2408-2417.

125. Maus MV, Grupp SA, Porter DL, June CH. Antibody-modified T cells: CARs take the front seat for hematologic malignancies. *Blood*. 2014;123(17):2625-2635.

126. Lowenberg B. Introduction to the review series on leukemic stem cells. *Blood*. 2017;129(12):1567.

127. Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. *Nature*. 2013;500(7463):415-421.

128. Jordan CT. Cancer stem cell biology: from leukemia to solid tumors. *Curr Opin Cell Biol*. 2004;16(6):708-712.

129. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell*. 2015;160(1-2):48-61.

130. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science*. 2015;348(6230):69-74.

131. Fontana MC, Marconi G, Feenstra JDM, et al. Chromothripsis in acute myeloid leukemia: Biological features and impact on survival. *Leukemia*. 2017.

132. Knudson AG, Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A*. 1971;68(4):820-823.

133. Cline MJ. The molecular basis of leukemia. *N Engl J Med*. 1994;330(5):328-336.

134. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol*. 2002;3(11):991-998.

135. Shankaran V, Ikeda H, Bruce AT, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 2001;410(6832):1107-1111.

136. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. 2012;481(7382):506-510.

137. Garrido F, Algarra I. MHC antigens and tumor escape from immune surveillance. *Adv Cancer Res*. 2001;83:117-158.

138. McGranahan N, Rosenthal R, Hiley CT, et al. Allele-Specific HLA Loss and Immune Escape in Lung Cancer Evolution. *Cell*. 2017;171(6):1259-1271 e1211.

139. Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. *Immunol Today*. 2000;21(9):455-464.

140. Shukla SA, Rooney MS, Rajasagi M, et al. Comprehensive analysis of cancer-associated somatic mutations in class I HLA genes. *Nat Biotechnol*. 2015;33(11):1152-1158.

141. Raghavan M, Smith LL, Lillington DM, et al. Segmental uniparental disomy is a commonly acquired genetic event in relapsed acute myeloid leukemia. *Blood*. 2008;112(3):814-821.

142. Vago L, Perna SK, Zanussi M, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med*. 2009;361(5):478-488.

143. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today*. 1990;11(7):237-244.

144. Grosse-Wilde H, Doxiadis I, Vogeler U, et al. An HLA lost mutation may lead to leukemic relapse of recipient type six years after bone marrow transplantation. *Haematol Blood Transfus*. 1987;30:571-573.

145. Vago L, Toffalori C, Ciceri F, Fleischhauer K. Genomic loss of mismatched human leukocyte antigen and leukemia immune escape from haploidentical graft-versus-leukemia. *Semin Oncol*. 2012;39(6):707-715.

146. Kobayashi S, Kikuta A, Ito M, et al. Loss of mismatched HLA in myeloid/NK cell precursor acute leukemia relapse after T cell-replete haploidentical hematopoietic stem cell transplantation. *Pediatr Blood Cancer*. 2014;61(10):1880-1882.

147. McCurdy SR, Iglehart BS, Batista DA, et al. Loss of the mismatched human leukocyte antigen haplotype in two acute myelogenous leukemia relapses after haploidentical bone marrow transplantation with post-transplantation cyclophosphamide. *Leukemia*. 2016;30(10):2102-2106.

148. Stolzel F, Hackmann K, Kuithan F, et al. Clonal evolution including partial loss of human leukocyte antigen genes favoring extramedullary acute myeloid leukemia relapse after matched related allogeneic hematopoietic stem cell transplantation. *Transplantation*. 2012;93(7):744-749.

149. Tamaki H, Fujioka T, Ikegame K, et al. Different mechanisms causing loss of mismatched human leukocyte antigens in relapsing t(6;11)(q27;q23) acute myeloid leukemia after haploidentical transplantation. *Eur J Haematol*. 2012;89(6):497-500.

150. Cieri N, Greco R, Crucitti L, et al. Post-transplantation Cyclophosphamide and Sirolimus after Haploidentical Hematopoietic Stem Cell Transplantation Using a Treosulfan-based Myeloablative Conditioning and Peripheral Blood Stem Cells. *Biol Blood Marrow Transplant*. 2015;21(8):1506-1514.

151. Crucitti L, Crocchiolo R, Toffalori C, et al. Incidence, risk factors and clinical outcome of leukemia relapses with loss of the mismatched HLA after partially incompatible hematopoietic stem cell transplantation. *Leukemia*. 2015;29(5):1143-1152.

152. Imus PH, Blackford AL, Bettinotti M, et al. Major Histocompatibility Mismatch and Donor Choice for Second Allogeneic Bone Marrow Transplantation. *Biol Blood Marrow Transplant*. 2017;23(11):1887-1894.

153. Vago L, Ciceri F. Choosing the Alternative. *Biol Blood Marrow Transplant*. 2017;23(11):1813-1814.

154. Gendzekhadze K, Gaidulis L, Senitzer D. Chimerism testing by quantitative PCR using Indel markers. *Methods Mol Biol*. 2013;1034:221-237.

155. Trenschel R, Ross S, Husing J, et al. Reduced risk of persisting cytomegalovirus pp65 antigenemia and cytomegalovirus interstitial pneumonia following allogeneic PBSCT. *Bone Marrow Transplant*. 2000;25(6):665-672.

156. Bustin SA. Why the need for qPCR publication guidelines?--The case for MIQE. *Methods*. 2010;50(4):217-226.

157. Voss G, Nick S, Stahl-Hennig C, Ritter K, Hunsmann G. Generation of macaque B lymphoblastoid cell lines with simian Epstein-Barr-like viruses: transformation procedure, characterization of the cell lines and occurrence of simian foamy virus. *J Virol Methods*. 1992;39(1-2):185-195.

158. Toffalori C, Cavattoni I, Deola S, et al. Genomic loss of patient-specific HLA in acute myeloid leukemia relapse after well-matched unrelated donor HSCT. *Blood*. 2012;119(20):4813-4815.

159. Lange V, Bohme I, Hofmann J, et al. Cost-efficient high-throughput HLA typing by MiSeq amplicon sequencing. *BMC Genomics*. 2014;15:63.

160. Gonzalez-Galarza FF, Takeshita LY, Santos EJ, et al. Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. *Nucleic Acids Res*. 2015;43(Database issue):D784-788.

161. Wiseman DH. Donor cell leukemia: a review. *Biol Blood Marrow Transplant*. 2011;17(6):771-789.

162. Ahci M, Stempelmann K, Buttkereit U, et al. Clinical Utility of Quantitative PCR for Chimerism and Engraftment Monitoring after Allogeneic Stem Cell Transplantation for Hematologic Malignancies. *Biol Blood Marrow Transplant*. 2017;23(10):1658-1668.

163. Ahci M, Toffalori C, Bouwmans E, et al. A new tool for rapid and reliable diagnosis of HLA loss relapses after HSCT. *Blood*. 2017;130(10):1270-1273.

164. Senitzer D GL. Short tandem repeat analysis of engraftment in allogeneic stem cell transplantation *ASHI Quarterly* 2001(25):49–54.

165. Karasawa M, Yamane A, Mitsui T, et al. Long-term persistence of host cells detected by X-chromosome gene-based assay in patients undergoing gender-mismatched hematopoietic stem cell transplantation. *Am J Hematol*. 2005;80(2):101-105.

166. Lugthart G, van Ostaijen-Ten Dam MM, Jol-van der Zijde CM, et al. Early cytomegalovirus reactivation leaves a specific and dynamic imprint on the reconstituting T cell compartment long-term after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2014;20(5):655-661.

167. Suessmuth Y, Mukherjee R, Watkins B, et al. CMV reactivation drives posttransplant T-cell reconstitution and results in defects in the underlying TCRbeta repertoire. *Blood*. 2015;125(25):3835-3850.

168. Reddehase MJ. Mutual Interference between Cytomegalovirus and Reconstitution of Protective Immunity after Hematopoietic Cell Transplantation. *Front Immunol*. 2016;7:294.

169. Ito S, Pophali P, Co W, et al. CMV reactivation is associated with a lower incidence of relapse after allo-SCT for CML. *Bone Marrow Transplant*. 2013;48(10):1313-1316.

170. Hourigan CS. Haplo, we have a problem. *Blood*. 2017;130(10):1180.

171. Fleischhauer K, Shaw BE, Gooley T, et al. Effect of T-cell-epitope matching at HLA-DPB1 in recipients of unrelated-donor haemopoietic-cell transplantation: a retrospective study. *Lancet Oncol*. 2012;13(4):366-374.

172. Rutten CE, van Luxemburg-Heijs SA, Griffioen M, et al. HLA-DP as specific target for cellular immunotherapy in HLA class II-expressing B-cell leukemia. *Leukemia*. 2008;22(7):1387-1394.

173. Shaw BE, Russell NH. Treatment options for the management of acute leukaemia relapsing following an allogeneic transplant. *Bone Marrow Transplant*. 2008;41(5):495-503.

174. Fleischhauer K, Shaw BE. HLA-DP in unrelated hematopoietic cell transplantation revisited: challenges and opportunities. *Blood*. 2017;130(9):1089-1096.

175. Marty R, Kaabinejadian S, Rossell D, et al. MHC-I Genotype Restricts the Oncogenic Mutational Landscape. *Cell*. 2017;171(6):1272-1283 e1215.

9. Supplementary

Table 9.1: HLA-A, C, DPB1 typing of positive and negative reference DNA.

	A*		C*		DPB1*	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
ECACC[1]						
BM21	01:01		17:01		10:01	
VAVY	01:01		07:01		01:01	
AMA	02:01	26:07	02:02	03:04	n.d.	n.d.
SLE005	02:01		03:04		03:01	
CB	02:01	03:01	07:01		19:01	
KY	02:06	24:02	01:01		04:02	05:01
EHM	03:01		04:01		04:01	04:02
H0301	03:01		08:02		05:01	
AKIBA	24:02		12:02		09:01	09:01
KAS116	24:02		12:03		13:01	13:01
MGAR	26:01		07:01		04:01	
MOU	29:02		16:01		02:01	
DUCAF	30:02		05:01		02:02	
TEM	66:01		12:03		04:01	
CML	01:01	03:01	n.d.	n.d.	04:01	14:01
OSR[2,3]						
OSR#01	01:01	02:01	06:02	16:01	04:01	13:01
OSR#02	01:01	02:01	06:02		02:01	04:01
OSR#03	01:01	03:01	02:02	06:02	03:01	04:01
OSR#04	01:01	11:01	04:01	06:02	03:01	04:02
OSR#05	02:01	24:02	02:02	15:02	04:01	
OSR#06	02:01	30:02	04:01	14:02	04:01	09:01
OSR#07	02:01	68:01	07:02	04:01	04:01	10:01
OSR#08	02:01	68:01	01:02	02:02	04:01	04:02
OSR#09	02:01	68:01	04:01	07:01	02:01	04:02
OSR#10	03:01	25:01	07:02	08:02	02:01	04:01
OSR#11	03:01	26:01	12:03	04:01	02:01	10:01
OSR#12	03:01	32:01	03:03	05:01	04:01	13:01
OSR#13	03:01	68:01	07:02	12:03	04:01	04:02
OSR#14	03:01	68:01	07:02	12:03	04:01	04:02
OSR#15	03:01	68:01	04:01	07:01	04:01	05:01
OSR#16	11:01	24:02	15:02		02:01	04:01
OSR#17	11:01	25:01	06:01	12:01	02:01	
OSR#18	23:01	24:01	07:01		04:01	14:01
OSR#19	23:01	30:01	04:01	06:01	02:01	
OSR#20	24:02		07:01		04:02	10:01
OSR#21	24:02		04:04	07:02	04:02	02:01
OSR#22	25:01	26:01	04:01	12:01	02:01	04:02
OSR#23	33:01	66:01	08:08	17:03	04:01	17:01
OSR#24	68:02	69:01	01:02	08:02	04:02	04:02

Table 9.1 (cont.).

	A*		C*		DPB1*	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
UKE[2,3]						
UKE#01	01:01	02:01	03:04	07:01	04:01	04:02
UKE#02	01:01	02:06	n.d.	n.d.	01:01	03:01
UKE#03	01:01	03:01	n.d.	n.d.	01:01	13:01
UKE#04	01:01	26:01:00	06:02	12:03	n.d.	n.d.
UKE#05	02:01		n.d.	n.d.	03:01	04:02
UKE#06	02:01		03:04	07:01	n.d.	n.d.
UKE#07	02:01		04:01	14:01	04:01	15:01
UKE#08	02:01	03:01	06:01	07:01	02:01	11:01
UKE#09	02:01	03:01	04:06		n.d.	n.d.
UKE#10	02:01	30:01	03:01	05:01	n.d.	n.d.
UKE#11	02:01	03:01	01:01	03:01	n.d.	n.d.
UKE#12	03:01	03:01	03:04	07:01	04:01	05:01
UKE#13	03:01	24:01	03:01	07:01	04:01	11:01
UKE#14	03:01	29:02	03:03	16:01	04:01	19:01
UKE#15	11:01	25:01	05:01	12:03	n.d.	n.d.
UKE#16	24:02:00	30:01	n.d.	n.d.	04:01	
UKE#17	25:01:00	26:01	03:02	04:01	02:01	04:01
UKE#18	n.d.	n.d.	n.d.	n.d.	01:01	03:01
UKE#19	n.d.	n.d.	n.d.	n.d.	01:01	11:01
UKE#20	n.d.	n.d.	n.d.	n.d.	02:01	03:01
UKE#21	n.d.	n.d.	n.d.	n.d.	02:01	04:02
UKE#22	n.d.	n.d.	n.d.	n.d.	02:01	04:02
UKE#23	n.d.	n.d.	n.d.	n.d.	02:01	11:01
UKE#24	n.d.	n.d.	n.d.	n.d.	04:01	14:01
UKE#25	n.d.	n.d.	n.d.	n.d.	04:01	14:01
UKE#26	n.d.	n.d.	n.d.	n.d.	04:02	13:01

[1]HLA typed B lymphoblastoid cell lines (BLCLs) from the European Collection of Animal Cell Cultures (ECACC). [2]BLCLs or peripheral blood mononuclear cells (PBMC) from healthy HLA typed individual from OSR or UKE. (nd) no data available.

Table 9.2: Concept of the dual indexing PCR

	Forward (P5, Index2)
Outer Primer	AATGATACGGCGACCACCGAGATCTACAC**<INDEX2>ACACTCTTTCCCTACACGA**
Inner Primer	**ACACTCTTTCCCTACACGA**CGCTCTTCCGATCT<Target Specific Sequence>
	Reverse (P7, Index1)
Outer Primer	CAAGCAGAAGACGGCATACGAGAT**<INDEX1>GTGACTGGAGTTCAGACGTG**
Inner Primer	**GTGACTGGAGTTCAGACGTG**TGCTCTTCCGATCT<Target Specific Sequence>

Amplicon sequences include inner and outer primers, index sequences for index 1 and index 2 as well as the genomic target sequence.

Table 9.3: HLA-A, B, DRB1 typing of reference DNA.

	A*		B*		DRB1*	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
DNA						
UKE#01[1]	01:01:01:01	02:01:01:01	08:01:01	40:01:01	03:01:01:01	15:01:01:01
UKE#02[1]	01:01:01:01	02:06:01	08:01:01		03:01:01:01	
UKE#03[1]	01:01:01:01	03:01:01:01	07:02:01	08:01:01	03:01:01:01	15:01:01:01
UKE#07[1]	02:01:01:01		44:02:01:01	49:01:01	01:01:01	11:01:01
UKE#10[1]	02:01:01:01	03:01:01:01	07:02:01	13:02:01	07:01:01:01	
UKE#12[1]	03:01:01:01		18:01:01:01	40:01:01	09:01:02	13:02:01
UKE#16[1]	24:02:01:01	30:01:01	08:01:01	55:01:01	03:01:01:01	14:01:01
UKE#17[1]	25:01:01	26:01:01	35:01:01:01	58:01:01	03:01:01:01	13:02:01
UKE#28[2]	24:02:01:01		51:01:01:01	52:01:01:01	15:01:01:01	15:02:01
UKE#29[2]	11:01:01:01	24:02:01:01	15:02:01	55:01:01	14:05:01	15:01:01:01
UKE#30[2]	02:01:01:01	11:01:01:01	35:01:01:01	37:01:01	11:01:01	15:01:01:01
UKE#31[2]	01:01:01:01	24:02:01:01	08:01:01	44:02:01:01	03:01:01:01	13:01:01
UKE#32[2]	01:01:01:01	02:01:01:01	13:02:01	35:01:01:01	12:01:01	13:02:01
UKE#33[2]	01:01:01:01		40:01:01		11:01:01	
UKE#34[2]	01:01:01:01	31:01:03	08:01:01	40:01:01	03:01:01:01	07:01:01:01
UKE#35[2]	01:01:01:01	03:01:01:01	08:01:01	27:05:02	03:01:01:01	04:01:01
UKE#36[2]	01:02	66:08	58:01:01		13:01:01	13:02:01
UKE#37[2]	24:02:01:01	32:01:01	13:02:01	51:01:01:01	07:01:01:01	11:01:01
UKE#38[2]	24:02:01:01		51:01:01:01		01:01:01	
UKE#39[2]	26:01:01		08:01:01		15:01:01:01	
UKE#40[2]	02:01:01:01		40:01:01		13:02:01	
UKE#41[2]	01:01:01:01		08:01:01		03:01:01:01	

[1]HLA typed B lymphoblastoid cell lines (BLCLs) from the European Collection of Animal Cell Cultures (ECACC) see Supplementary Table 9.1. [2]BLCLs or peripheral blood mononuclear cells (PBMC) from healthy HLA typed individual from UKE.

10. Danksagung

An erster Stelle gilt mein Dank meiner Doktormutter, Frau Prof. Dr. med. Katharina Fleischhauer, für ihre Unterstützung während der gesamten Phase meiner Promotion. Danke für die zahlreichen und unermüdlichen Fachgespräche, Ratschläge und Anmerkungen, die mich auf dem Weg zur fertigen Promotionsschrift immer wieder neue Aspekte und Ansätze entdecken ließen. Danke für die gebotenen Chancen zur Stärkung meiner Erkenntnisse über die Stammzelltransplantation, Immunogenetik und die Welt des HLA, vor allem auch durch die Teilnahme an diversen Konferenzen.

Außerdem gilt mein Dank Herrn Dr. Luca Vago und Herrn Dr. Pietro Crivello, die mich während der Jahre stets durch zielführende Diskussionen und anhaltende Hilfestellung begleitet und unterstützt haben.

Frau Karin Stempelmann, die mir fortwährend Hilfestellung bei der Durchführung praktischer Arbeiten und Experimente gegeben hat. Zusammen mit Frau Elke Stratmann haben mich auch unsere vielen nicht-wissenschaftlichen und motivierenden Gespräche bei meiner Arbeit sehr unterstützt. Danken möchte ich auch meinem ehemaligen Arbeitskollegen Swen Engelmann für die schönen Erinnerungen und die Mühe beim Korrekturlesen der vorliegenden Dissertation.

Allen Mitarbeitern der Transfusionsmedizin und der Knochenmarkstransplantation der Universitätsklinik Essen, vor allem Herrn Prof. Dr. Dietrich W. Beelen, Herrn Dr. Heinold und Herrn Dr. Falko Heinemann, sowie allen Kooperationsmitarbeitern der Firma Gendx und der DKMS Life Science Lab, bin ich sehr dankbar für die gute, sowie die konstruktive und angenehme Zusammenarbeit.

Besonders möchte ich mich an dieser Stelle bei meiner Familie bedanken. Am meisten bei meinen Eltern F. und H. Ahci. Sie haben mir jede Art von Rückhalt und Kraft gegeben, sodass ich meinem Traum Schritt für Schritt Folge leisten konnte. Bei meinen Geschwistern B., S. und S. Ahci, die fest an mich, die große Schwester, geglaubt haben. Insbesondere aber auch bei meinen Opas MÖ und SA sowie meiner, während der Endphase verstorbenen Omas NÖ und HA für die Stärkung und Motivation, sowie für das stets offene Ohr für meine Gefühle und Gedanken.

11. Curriculum Vitae - CV

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12.Publications

PEER-REVIEWED PUBLICATIONS

Müberra Ahci, Pietro Crivello, Karin Stempelmann, Ulrike Buttkereit, Nona Shayegi, Andreas Heinold, Falko M. Heinemann, Peter A. Horn, Dietrich W. Beelen, Katharina Fleischhauer. “Subtle changes in host chimerism after unrelated hematopoietic cell transplantation can be monitored by quantitative PCR”. *Biology of Blood and Marrow Transplantation* 2017 Oct;23(10):1658-1668. doi: 10.1016/j.bbmt.2017.05.031. Epub 2017 Jun 8.

Journal Impact Factor 2016: 4.704

Müberra Ahci*, Cristina Toffalori*, Evelien Bouwmans, Pietro Crivello, Chiara Brambati, Cinzia Pultrone, Karin Stempelmann, Douglas Bost, Benedetta Mazzi, Dietrich W. Beelen, Fabio Ciceri, Wietse Mulder, Katharina Fleischhauer*, Luca Vago*. “A new tool for rapid and reliable diagnosis of HLA Loss relapses after HSCT”. (*equal contribution) *Blood* 2017 2017 Sep 7;130(10):1270-1273. doi: 10.1182/blood-2017-05-784306. Epub 2017 Jun 27.

Commentary “Haplo, we have a problem” on Ahci and Toffalori et al. by Cristopher S. Hourigan *Blood* 2017 Blood. 2017 Sep 7;130(10):1180. doi: 10.1182/blood-2017-07-795062.

Journal Impact Factor 2016: 13.164

Esteban Arrieta-Bolaños, Pietro Crivello, Maximilian Metzing, Thuja Meurer, **Müberra Ahci**, Julie Rytlewski, Marissa Vignali, Erik Yusko, Peter Van Balen, Peter A. Horn, Fred Falkenburg and Katharina Fleischhauer “Alloreactive TCR diversity against structurally similar or dissimilar HLA-DP antigens assessed by deep sequencing” Front. Immunol. 31 Jan 2018. doi: 10.3389/fimmu.2018.00280

Journal Impact Factor 2016: 6.429

UNDER SUBMISSION

Pietro Crivello, **Müberra Ahci**, Esteban Arrieta-Bolanos, Andreas Heinold, Vinzenz Lange, J.H. Frederik Falkenburg, Peter A. Horn, Katharina Fleischhauer, Stefan Heinrichs “Multiple knockout of classical HLA class II β-chains by CRISPR/Cas9 genome editing driven by a single guide RNA”. Submitted to Journal of Immunology

Journal Impact Factor 2016: 5.185

IN PREPARATION

Müberra Ahci, Cristina Toffalori, Luca Vago, Katharina Fleischhauer. “Chimerism Follow-up after allogeneic hematopoietic stem cell transplantation: state of the art and significance”. To be submitted to *HLA* (invited Review Article).

Maximilian Metzing, Pietro Crivello, Esteban Arrieta-Bolaňos, **Müberra Ahci,** Thuja Meurer, Kees van Bergen, Peter van Balen, Peter A. Horn, Nils v. Neuhoff, Dietrich W. Beelen, Frederik Falkenburg, Katharina Fleischhauer. “HLA-DM mediates permissiveness of T-cell alloreactivity to HLA-DPB1”. To be submitted to *Immunity*.

Essen, 04.04.2018

M.sc. Müberra Ahci

13.Eidesstattliche Erklärungen

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Innovative approaches to the detection of HLA immune escape leukemia relapse after allogeneic hematopoietic stem cell transplantation“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Müberra Ahci befürworte.

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Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegeben Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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