Noninherited Maternal Human Leukocyte Antigens: Donor Availability and Clinical Outcome in Unrelated Cord Blood Transplantation

Leonie Powley

Division of Infection and Immunity, UCL

&

Histocompatibility and Immunogenetics Research Group, NHSBT

A thesis submitted to University College London for the degree of

Doctor of Philosophy

Declaration

I, Leonie Powley. confirm that the work presented in this thesis is my own.

Where information has been derived from other sources, I confirm that this has

been indicated in the thesis.

Declaration

Abstract

Foetal exposure to semi-allogeneic cells from maternal microchimaerism (MMc) has been associated with the development of tolerance towards noninherited maternal antigens (NIMA) through a regulatory T cell response. This concept can be exploited in the selection of permissible Human Leukocyte Antigen (HLA) mismatches in cord blood (CB) transplantation (CBT) due to the availability of maternal samples for HLA typing and the identification of NIMA. CB virtual phenotypes (VPs) were generated by the substitution of 1-3 CB HLA (*HLA-A*, *-B* and/or *–DRB1*) for the corresponding NIMA, to permit the identification of virtual full HLA matches (VFM: 5/6 + 1 NIMA, 4/6 + 2 NIMA or 3/6 + 3 NIMA) for 457 patients. Maternal HLA typing for 4,671 CB donors resulted in the generation of 66,225 VPs and 52,875 of these were unique. When combined with the inherited phenotypes of 21,020 CB donors, the total unique phenotypes to increased to 65,046. VFMs, with adequate cell dose, doubled the cumulative availability of a matched donor for European Caucasoid patients and tripled the availability for patients of other ethnicities. Analyses of NIMA matching and clinical outcomes were performed for 198 transplants but was statistically underpowered to detect an association.

High levels of MMc have been associated with transplantation tolerance. HLA q-PCR assays with 0.01% sensitivity were optimised. MMc was detected in 27% of 96 samples tested. MMc was more frequent in CB from earlier gestational time points and appeared to be associated with bi-directional maternal-foetal HLA compatibility.

The incorporation of NIMA in CB donor selection therefore increases the donor pool available to patients, particularly ethnic minorities, requiring CBT.

Acknowledgements

I would like to thank Dr Cristina Navarrete for your kindness, guidance, time, encouragement, support and for providing the freedom and confidence to follow my initiative. Cristina you were a fantastic mentor and role model. Now it is finally time for you to enjoy some quality time with your grandchildren!

Prof Ron Chakraverty, for your support.

Dr Colin Brown, for your counselling, guidance, advice and time. John Ord and Ellie Curnow, for your expert technical knowledge. All the other NHSBT staff involved in this project, without your cooperation much of this work would not have been possible.

Prof John van Rood and Dr Michael Eikmans, for your expert advice.

Sarah Peacock and Dr Craig Taylor, for your support and patience whilst I was finishing this thesis. Jackie Wyse, for your brilliant proof-reading skills.

My parents, Louise Knight and Konrad Powley, for encouraging me to follow this path, for never doubting me and for their selflessness. My brother and sister, Harry and Rosie. Without the support and love of my family I would not be where I am today.

My friend, Dr Alison Niewiarowska, for sharing your ability to quickly understand a subject you're not familiar with and come up with a valid critique. Lyn, for having a fantastic attitude to life. And to Borris of course!

This thesis is dedicated to my Dad

KONRAD POWLEY

who taught me the power of education.

Contents

List of figures

List of Tables

List of Abbreviations

µl	Microliter
aGvHD	Acute graft-versus-host disease
APC	Antigen presenting cell
ASI	Asian
ATG	Anti-thymocyte globulin
BBMR	British Bone Marrow Registry
BLK	Black
BM	Bone marrow
BMDW	Bone Marrow Donors Worldwide
BMT	Bone marrow transplantation
CAU	Caucasian
CB	Cord blood
CBB	Cord blood bank
CBT	Cord blood transplant
CBU	Cord blood unit
CD	Classification determinant
cGvHD	Chronic graft-versus-host disease
CIBMTR	Centre for International Blood and Marrow Transplant Research
C_q	Cycle quantitation
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
E	Efficiency
EBMT	European Blood and Marrow Transplant Group
EC	European Caucasoid
EM	Expectation-maximisation
GvH	Graft-versus-host
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukaemia
H&I	Histocompatibility and Immunogenetics
HCK	Human Cell Kinase
HLA	Human Leukocyte Antigen

HPC	Haematopoietic progenitor cell
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
HvG	Host-versus-graft
IBD	Identical by descent
IFM	Inherited full match
IFN-γ	Interferon gamma
IL	Interleukin
IMA	Inherited maternal antigen
InDel	Insertion Deletion
IP	Inherited phenotype
IPA	Inherited paternal antigen
KIR	Killer cell immunoglobulin-like receptor
LD	Linkage disequilibrium
LDR	Linear dynamic range
LN	Lymph node
m-AAQ	Membrane alloantigen acquisition
MAC	Myeloablative conditioning
MHC	Major Histocompatibility Complex
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MIX	Mixed
ml	Millilitre
MLR	Mixed lymphocyte reaction
MM	Mismatch
MMc	Maternal microchimaerism
MUD	Matched unrelated donor
ng	Nano gram
NHS-CBB	NHS Cord Blood Bank
NIMA	Noninherited maternal antigen
NIMA-	Non NIMA matched, HLA mismatched transplant
NIMA+	NIMA matched, HLA mismatched transplant
NIPA	Noninherited paternal antigen

NK	Natural killer
NMDP	National Marrow Donor Program
NTC	Non-template control
NYCB	New York Cord Bank
OE	Other ethnicity
OS	Overall survival
OTH	Other
PB	Peripheral blood
PCR	Polymerase chain reaction
pmol	Picomole
q-PCR	Quantitative PCR
RIC	Reduced intensity conditioning
SCID	Severe combined immunodeficiency
SEA	South and East Asian
SSP	Sequence specific primer
TBI	Total body irradiation
TCD	T cell depletion
TCR	T cell receptor
TGF-β	Transforming growth factor beta
T_H	T helper
T_m	Melting temperature
TNC	Total nucleated cell
Treg	Regulatory T cell
TRM	Transplant related mortality
UD	Unrelated donor
UNK	Unknown
VFM	Virtual full match
VP	Virtual phenotype

Chapter 1 General introduction and thesis aims

1.1 An overview of the immune system

The immune system is tasked with the recognition and elimination of infectious agents, whilst distinguishing these pathogens from self. It comprises a set of cells, tissues and molecules and in vertebrates, is broadly categorised into two arms: innate and adaptive immunity. Innate immunity offers a first line of defence by recognising highly conserved molecular motifs within broad classes of microbes. Binding between these molecular motifs and germ line encoded pattern recognition receptors, trigger signalling cascades that result in the phagocytosis and/or direct killing of pathogens and the recruitment of immune cells to the site of infection through the process of inflammation. If a pathogen is not eliminated or contained by these mechanisms, the adaptive immune response is initiated. This highly specialised, systemic response is mediated primarily by T and B lymphocytes and unlike innate immunity, results in immunological memory, allowing an enhanced response for subsequent encounters with the same pathogen. Once activated, B lymphocytes mature into antibody producing plasma cells which aid phagocytosis by innate immune cells and activate the complement system. T lymphocytes comprise T helper (T_H) and cytotoxic T lymphocytes (CTLs), which function to help B cells make antibodies and kill viral infected cells, respectively. Unlike innate immune cells, T and B lymphocytes express a highly diverse receptor repertoire that can recognise a variety of molecular structures. Receptors specific to particular antigens are clonally distributed on individual lymphocytes. T cells are themselves unable to directly recognise the whole antigen but instead bind processed antigenic peptides in the context of the Major Histocompatibility Complex (MHC) molecules.

1.2 Major histocompatibility complex

1.2.1 Discovery of the MHC and early transplantation attempts

The MHC is a complex genetic region that contains genes coding for molecules involved in the induction and regulation of immune responses. These were initially discovered because of their role in the rejection of organs and tissues. Early observations showed tumours could be transplanted between some strains of mice but were rejected between others, implying donor-recipient compatibility was genetically controlled. Snell and colleagues systematically bred strains of mice that were identical except from a single genetic region, allowing the effect of each region on tumour graft rejection to be examined (Snell 1948). He named the locus responsible the H locus (for histocompatibility), which he later realised was the same system encoding antigen II, an agglutinating antibody previously identified by Peter Gorer (Gorer 1938). Further work showed this region was actually composed of multiple genes closely linked together and was also present in other vertebrates. The human histocompatibility genes within the MHC are referred to as Human Leukocyte Antigens (HLA) and were discovered by the research groups of van Rood, Payne and Dausset in the 1950s from studies of leukocyte agglutinating antibodies in the sera of multiparous women and transfused patients (Dausset 1958, Payne 1957, Van Rood, *et al* 1958). These were characterised during the early international histocompatibility workshops that still exist today (Barker and Markmann 2013).

The large number of burns victims from the First and Second World Wars and the failure of attempts to treat these patients with unrelated skin grafts propelled research into allograft transplantation. Medawar and Gibson found that, although

both autografts and allografts initially healed successfully, allografts were later rejected within two weeks. When a second allograft from the same donor was attempted, the graft rejected faster and Medawar postulated this was due to an immune response (Medawar 1944). Importantly, Medawar, Billingham and Brent later showed, in a seminal experiment, that if mice were exposed to allogeneic cells during foetal development, they were able to tolerate later skin grafts from this allogeneic donor as adults (Billingham, *et al* 1953). This formed the basis of immunological tolerance.

1.2.2   The MHC genetic locus

The MHC region is located on the short arm of chromosome 6 (6p21.31), spans approximately 4 megabases and is the most gene dense region of the human genome (Shiina, *et al* 2009). The MHC is organised into three clusters of genes whose gene products have a similar structure and function: class I, class II and class III **(Figure 1.1)**. At the telomeric end of the MHC is the Class I region, which contains the classical *HLA-A*, *-B* and *-C* alpha (α)-chain genes (the class I beta (β)-chain gene is located outside of the MHC on chromosome 15). Centromeric to this is the class II region, which contains the classical *HLA-DR*, *-DQ* and *–DP* genes. The α and β chain of class II molecules are both encoded in the MHC, designated A (e.g. *DRA*) and B (e.g. *DRB*), respectively. The A and B genes are organised into pairs and contribute to the same isotype (except for *HLA-DOA* and *-DOB*, which are separated by other genes). HLA-DR haplotypes are complex and contain a variable number of coding and pseudogenes. There are four *DRB* loci, *DRB1*, *DRB3*, *DRB4* and *DRB5*. *HLA-DRB1* is highly polymorphic and present in all haplotypes, whereas the others are present in only some haplotypes. Each *DRB*

gene encodes a functional β-chain that can pair with the DR α-chain, meaning a potential expression of four different class II molecules on the cell surface.

Figure 1.1 Simplified map of the genetic structure of the human MHC region (Abbas, *et al* 2015).
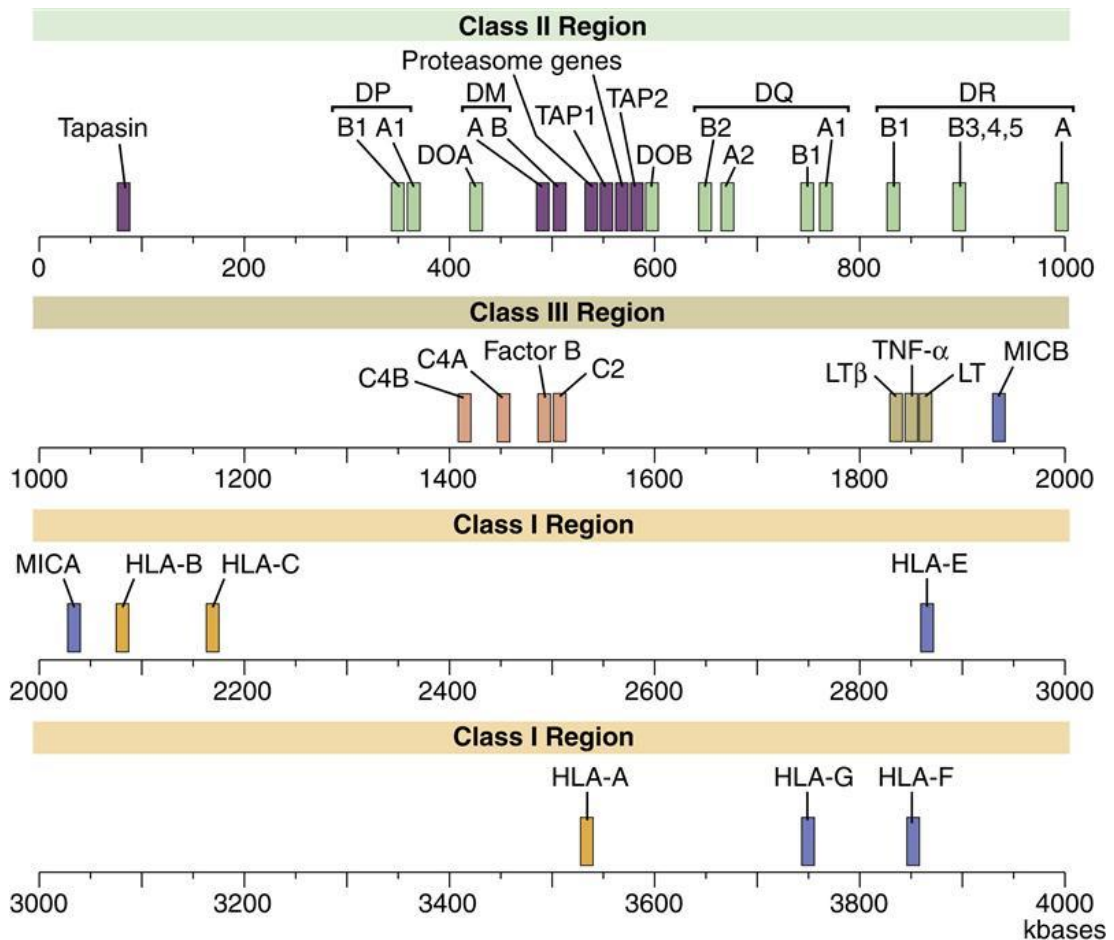
Among different ethnicities, certain combinations of HLA alleles occur more often on the same haplotype than would be expected on the basis of their individual gene frequencies alone, termed linkage disequilibrium (LD).

1.2.3   HLA protein structure

X-ray crystallography revealed HLA molecules are heterodimers consisting of two non-covalently associated glycoprotein chains, the α (heavy) chain and the β

(light) chain (Janeway, *et al* 2008). The two protein domains nearest to the membrane resemble an immunoglobulin domain and the two domains furthest away fold together to form a peptide binding cleft **(Figure 1.2)**.

Class I molecules consist of three α protein domains and a β-chain consisting of β_2-microglobulin. The peptide binding cleft is formed from the α-1 and α-2 helices and lies on a sheet of eight anti-parallel β strands. Class I molecules bind short peptides of 8 – 10 amino acids in length (Rudolph, *et al* 2006). The β-chain provides a platform for α-1 and α-2 and is required for stable cell surface expression.

Class II molecules differ in structure in that the β-1 domain of the β-chain forms part of the peptide binding cleft with the α-1 domain in a non-covalent association. The peptide-binding cleft is held into position by the both the α-2 and β-2 domains and unlike class I, both the α- and β-chains have transmembrane and intracytoplasmic domains. The peptide binding cleft of class II molecules permits the binding of longer peptides because the helices bordering the peptide are shorter and less curved, allowing the peptide to protrude from the ends of the groove (Rudolph, *et al* 2006).

Figure 1.2 Structure of class I and class II HLA molecules (Abbas*, et al* 2015).
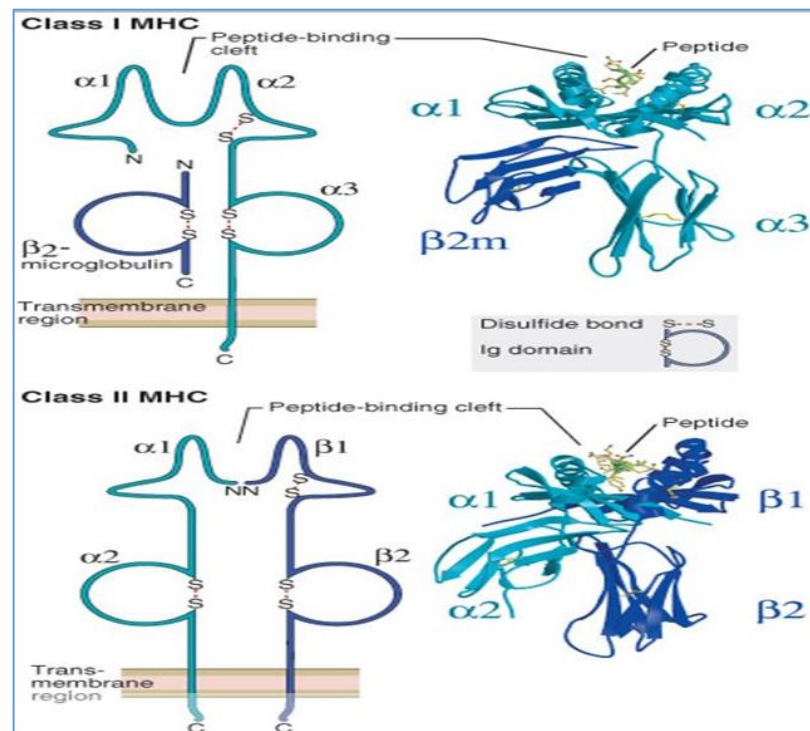
1.2.4 Function of HLA molecules

Class I and class II molecules differ in function and present peptides derived from separate antigen processing pathways. Class I molecules are expressed at variable levels on all nucleated cells and present intracellular-derived peptides, processed through the proteasome pathway, to the T cell receptor (TCR) of classification determinant (CD)8[+] CTLs **(Figure 1.3A)**. Class II molecules present extracellular-derived peptides, produced in acidic endocytic compartments that promote proteolysis **(Figure 1.3B),** to CD4^{+} T$_H$ cells. HLA class II molecules are constitutively expressed at low levels on antigen presenting cells (APCs), such as macrophages, immature dendritic cells (DCs) and B cells. Up-regulation in APCs can be induced by inflammatory cytokines and expression can also be up-regulated in otherwise class II negative cells, such as activated T cells and endothelial cells (Neefjes*, et al* 2011).

Figure 1.3 Antigen processing and presentation pathways of (A) Class I and (B) Class II HLA molecules (Neefjes, *et al* 2011).

(A)   **(B)**
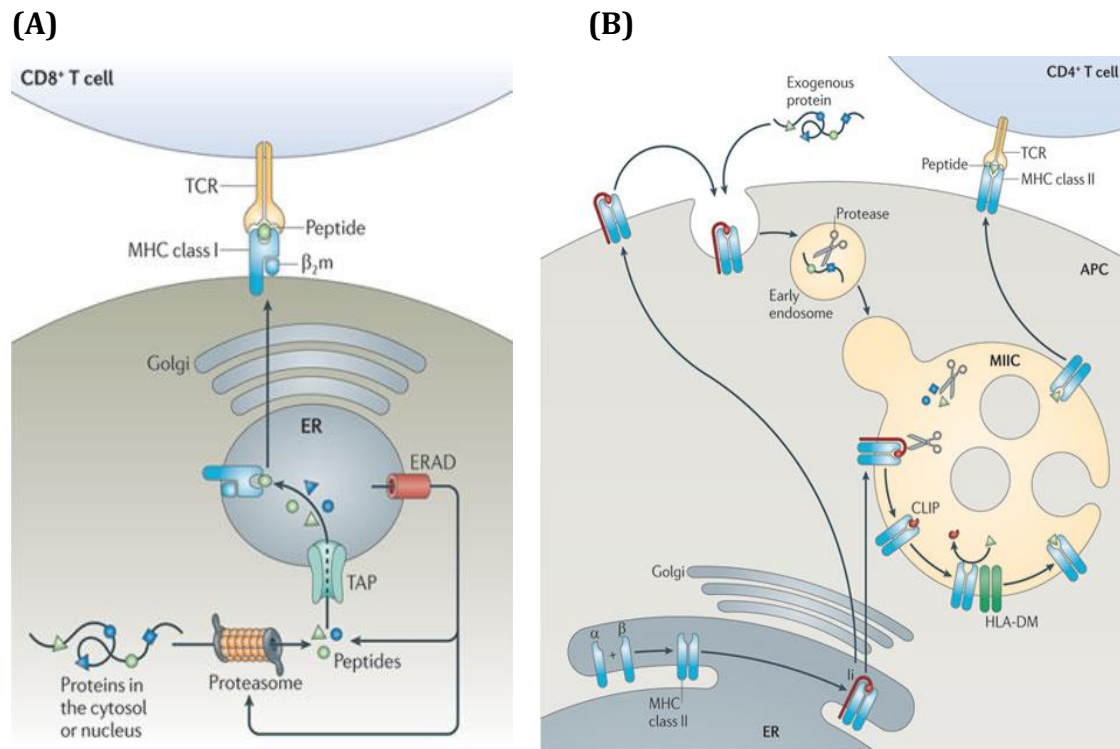
Abbreviations: APC, antigen presenting cell; β2m, β2-microglobulin; CLIP, class II-associated Ii peptide; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; Ii, invariant chain; MHC, major histocompatibility complex; MIIC; MHC class II compartment; TAP, transporter associated with antigen presentation; TCR, T cell receptor.

Peptide-HLA complexes are presented to T cells through the TCR and its co-receptor, CD3. Each T cell expresses a clonal TCR that is specific for a peptide-HLA complex, with more than 10^8 potential combinations achieved by somatic V(D)J recombination and gene conversion during early stages of development in the thymus **(see section 1.4.2.2)**. TCR binding to the peptide-HLA complex initiates a signalling cascade that determines T cell fate through regulating cytokine production, cell survival, proliferation and differentiation and

ultimately leads to the activation of effector function (Krogsgaard and Davis 2005).

TCRs exist predominantly as αβ heterodimers containing a variable and a constant region belonging to the immunoglobulin superfamily. There is a two-step model of TCR binding to peptide-HLA complexes, where the TCR is first able to bind the HLA α-helices and then efficiently "scan" for agonist peptide-HLA through substantial movement of the hypervariable region complementarity determining region 3 loops, the principle determinants of peptide specificity (Krogsgaard and Davis 2005). This causes a conformational change, known as an induced fit and the high energy contacts determine whether binding has enough stability to trigger T cell activation (Krogsgaard and Davis 2005). After docking and stabilisation, a signalling cascade is triggered through engagement of T cell co-receptors, CD3 and either CD4 (T_H cells) or CD8 (CTL cells) (Krogsgaard and Davis 2005).

1.2.5 Inheritance of the HLA genes

Family studies have shown that recombination in the HLA region is rare (less than 1%) (Lam, *et al* 2013) and therefore a complete set of HLA alleles on the same chromosome are usually inherited as an HLA haplotype **(Figure 1.4)**. Inheritance of HLA haplotypes is Mendelian, with an individual inheriting one haplotype from the mother (containing the inherited maternal antigens (IMA)) and one haplotype from the father (containing the inherited paternal antigens (IPA)). The HLA molecules of the mother and father that were not inherited are

known as the noninherited maternal antigens (NIMA) and the noninherited paternal antigens (NIPA), respectively.

Figure 1.4 Inheritance of HLA haplotypes. The child has inherited the maternal haplotype C (IMA: A3, B7, DR15) and the paternal haplotype A (IPA: A1, B8, DR17). Haplotype B (NIPA: A2, B44 and DR4) and D (NIMA: A24, B57, DR7) were not inherited from the father and mother, respectively.
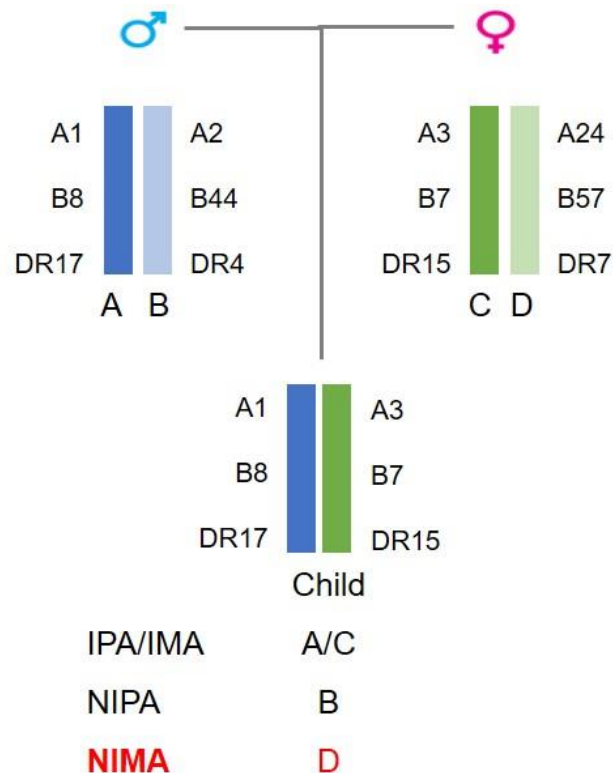
Abbreviations: IMA, inherited maternal antigen; IPA, inherited paternal antigen; NIMA, noninherited maternal antigen; NIPA, noninherited paternal antigen.

1.2.6 HLA polymorphism and evolution

The MHC is polygenic and it is thought to have evolved from repeated gene duplication and deletion events (Trowsdale 2011). Each individual has the potential to express six different functional classical class I and eight class II gene products, which constitutes the phenotype. The MHC is also the most polymorphic region in the human genome and therefore an individual is likely to be heterozygous at each locus. This balancing selection/ heterozygote advantage

offers two chances at an HLA locus to detect an infectious pathogen. Polymorphisms include point mutations, insertion/deletions and microsatellites and are found within the exons encoding the peptide binding region (class I: exons 2 and 3 encoding the α-1 and α-2 domains respectively; class II: exon 2 encoding the β-2 domain), whereas the exons encoding the protein domains involved in TCR and co-receptor interactions are generally conserved (Marsh, *et al* 1999). Many polymorphisms encode amino acid changes (nonsynonymous) and just one amino acid substitution can cause dramatic alterations in antigen binding affinity and consequently alter the efficiency of T cell activation (Khanna, *et al* 1999, Kubo, *et al* 1998).

1.3 Allogeneic haematopoietic stem cell transplantation

1.3.1 Purpose and indications for transplant

The primary aim of haematopoietic stem cell (HSC) transplantation (HSCT) is to re-establish haematopoietic and immune function in patients whose bone marrow (BM) or immune system is damaged or defective; or to repair single gene defects in metabolic disorders (Apperley, *et al* 2012). Prior to HSCT, a myeloablative conditioning (MAC) or reduced intensity conditioning (RIC) regimen is administered to provide sufficient immunoablation to make space for the graft, eradicate patient disease and prevent graft rejection (Apperley, *et al* 2012).

1.3.2   Post-transplantation events

1.3.2.1   Engraftment, immune reconstitution and infection

Following allogeneic HSCT patients experience a period of profound immunodeficiency prior to complete immune reconstitution, which is heightened through the use of post-transplant immunosuppression (Merindol, *et al* 2011) **(Figure 1.5)**. During this time there is a risk of opportunistic infections and viral induced malignancies (Merindol, *et al* 2011).

Figure 1.5 Kinetics of immune reconstitution and transplant-related complications in children following cord blood transplantation (Merindol, *et al* 2011).
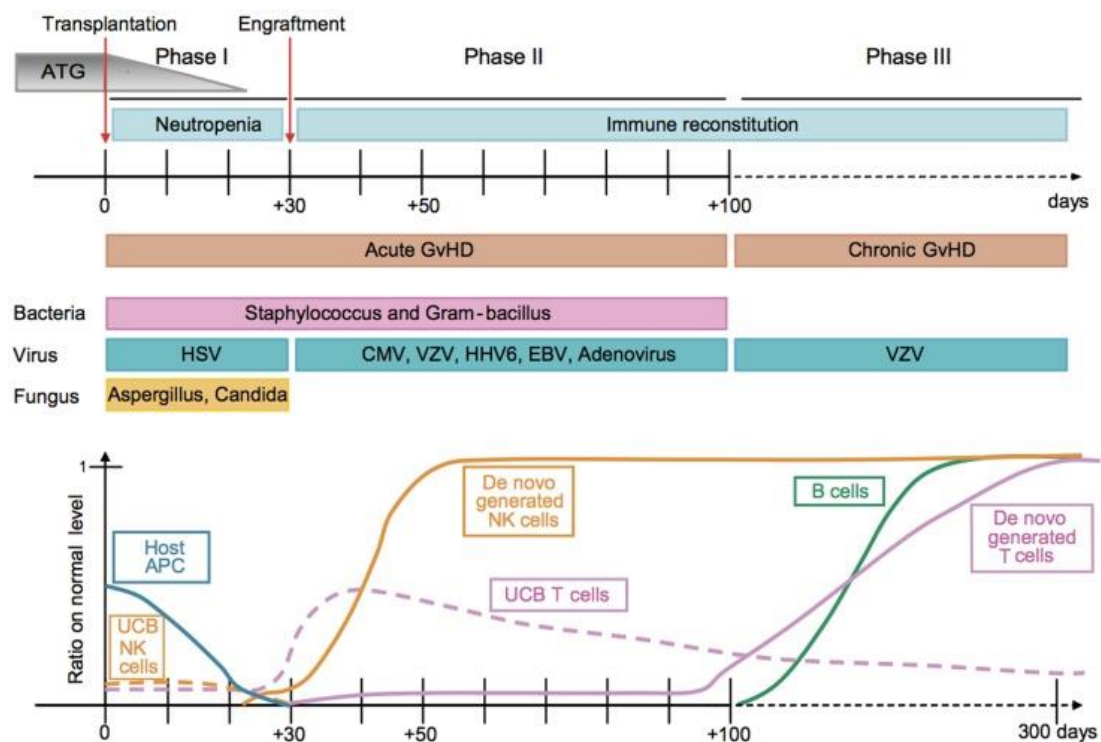
Abbreviations: APC, antigen presenting cell; ATG, anti-thymocyte globulin; CMV, Cytomegalovirus; EBV, Epstein-Barr Virus; GvHD, graft-versus-host disease; HHV6, Human Herpes Virus 6; HSV, Herpes Simplex Virus; NK, natural killer; UCB, umbilical cord blood; VZV, Varicella zoster virus.

1.3.2.2 GvHD and relapse

Graft-versus-host (GvH) disease (GvHD) is a major cause of morbidity and mortality after allogeneic HSCT and is classified into acute and chronic forms (Eapen, *et al* 2011b, Flomenberg, *et al* 2004, Furst, *et al* 2013, Jagasia, *et al* 2012, Kanda 2013, Lee, *et al* 2007, Woolfrey, *et al* 2011).The development of acute GvHD (aGvHD) is dependent on three requirements: 1) immunocompetent cells present in the graft, 2) recipient expression of tissue antigens that are absent in the donor and 3) the recipient must be unable eliminate the graft. Acute GvHD generally occurs within 100 days post-transplantation and occurs in three stages **(Figure 1.6)** (Ferrara, *et al* 2009). Chronic GvHD (cGvHD) generally occurs later and manifests *de novo* or as a progression from aGvHD and has a poorly understood pathophysiology (Ferrara, *et al* 2009). It is thought to be mediated by donor T cells but unlike aGvHD, tends to share features with autoimmune disease (Ferrara, *et al* 2009).

GvHD is closely correlated with the beneficial graft-versus-leukaemia (GvL) effect. For malignant disease, the optimal conditioning regimen should provide reduced relapse risk without an increased risk of GvHD (Apperley, *et al* 2012).

Figure 1.6 Pathophysiology of aGvHD. The recipient conditioning regimen damages tissues and causes the release of inflammatory cytokines and activation of host APCs (phase I). Host APCs activate donor T cells, leading to activation, proliferation, differentiation and migration of alloreactive donor T cells (phase II). This results in a complex cascade of multiple cellular (e.g. CTL) and inflammatory (e.g. TNF-α) effectors that further modulate each other's responses and ultimately leads to target organ damage. T cell effector mechanisms can be suppressed by Tregs (Ferrara, *et al* 2009).
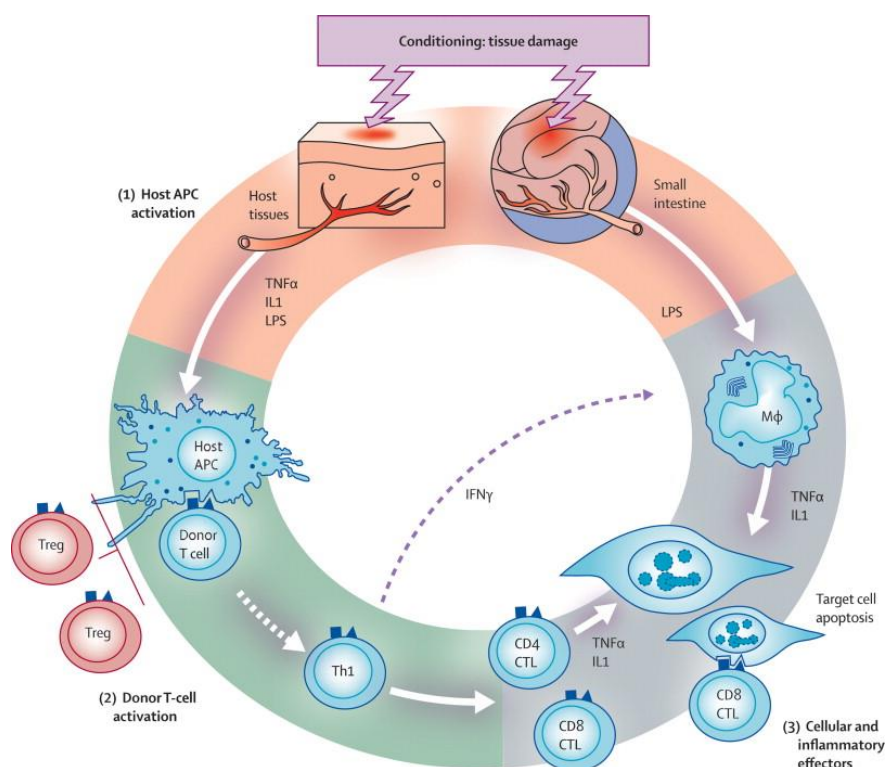
Abbreviations: APC, antigen presenting cell; CTL, cytotoxic T lymphocyte; IFN-γ, interferon-γ; IL-1, interleukin 1; LPS, lipopolysaccharide; Th1, T-helper 1 cell; TNF-α, transforming growth factor beta; Treg, regulatory T cell.

1.3.3 HSCT donors

HSCs can be harvested from BM or peripheral blood (PB) from different types of donors. HLA compatibility is the predominant determinant of the success of HSCT and the optimal donor is an HLA genotype identical sibling **(Table 1.1)** but more than two thirds of patients require an unrelated donor (UD), facilitated through searching altruistic volunteer registries (Passweg, *et al* 2016). In the absence of

an HLA matched UD (MUD, 10/10 based on *HLA-A*, *-B*, *-C*, *-DRB1* and *–DQB1* at allelic resolution), an HLA mismatched UD (9/10) can be utilised, albeit with detrimental clinical outcomes (Lee*, et al* 2007, Petersdorf 2008). Alternative donors include an HLA haploidentical donor (family member) or cord blood (CB), which are more rapidly available compared to adult UD (Lown and Shaw 2013).

Table 1.1 Related and unrelated donors for HSCT and their HLA matching criteria.

Type of HSCT donor	HLA matching
Related donor	
HLA identical sibling	Genotype matched – Patient and sibling share both HLA haplotypes.
Haploidentical donor	The patient and donor (parent or sibling) share one HLA haplotype, as shown from familial haplotype assignments.
Unrelated donor	
Adult donor	*HLA-A*, *-B*, *-C*, *–DRB1* and *–DQB1* matched at allelic resolution
Unrelated Cord Blood	Historically matched for *HLA-A* and *–B* at antigenic or intermediate resolution and *–DRB1* at allelic or high resolution. More recently matched for *HLA-A*, *-B*, *-C* and *–DRB1* at allelic resolution.

1.3.4   HLA matching and clinical outcome of transplantation

In adult donor HSCT, single and multiple HLA mismatches (MMs) have been shown to increase the risk of GvHD (*HLA-A*, *-B* and *-C*), transplant related mortality (TRM; *HLA-A*, *-C* and *-DRB1*) and result in an additive 9 - 10% increase in mortality (*HLA-A*, *-C* and *-DRB1*) (Lee*, et al* 2007, Petersdorf 2008). Historically, a major clinical advantage of CB was the less stringent HLA matching requirements due to the lower associated incidence and severity of aGvHD compared with BM/PB (Brunstein*, et al* 2010, Eapen*, et al* 2010, Eapen*, et al* 2007). This has been attributed to the lower lymphocyte content in CB grafts and

a limited response of CB naïve T cells activated by recipient alloantigen, possibly due to a pre-disposition towards tolerance (Mold, *et al* 2010), resulting in impaired cytokine production, limited cellular activation and a lack of clonal expansion of activated T cells (Brown and Boussiotis 2008). Although CB is associated with a lower incidence of GvHD, the risk of relapse is generally comparable between BM transplantation (BMT) and CB transplantation (CBT) in both children (Eapen, *et al* 2006, Eapen, *et al* 2007) and adults (Eapen, *et al* 2010, Takahashi, *et al* 2007).

An increasing accumulation of evidence supports matching at *HLA-C* (Eapen, *et al* 2014, Eapen, *et al* 2011a, Eapen, *et al* 2011b) and at allelic resolution for *HLA-A*, *-B*, *-C* and *–DRB1* in CBT (Kogler, *et al* 2005, Kurtzberg, *et al* 2008, Matsuno, *et al* 2009). Data indicates that 1 - 2 HLA allele MMs are acceptable but 3 - 5 allele MMs should be avoided (Eapen, *et al* 2014) and updated recommendations on the selection of cord blood units (CBUs) reflect these findings (Hough, *et al* 2016).

Clinical outcome data also suggest that not all HLA MMs have a comparable detrimental effect on clinical outcome (Kawase, *et al* 2009, Kawase, *et al* 2007). Permissible HLA MMs identified for adult donor HSCT are based on amino acid substitutions at key regions of the HLA molecule involved in peptide binding or TCR interactions (Kawase, *et al* 2009, Kawase, *et al* 2007). Given that the identification of a MUD remains a challenge, especially for ethnic minorities, elucidating the differential effects of allele MM combinations may further aid donor selection (Gragert, *et al* 2014). In CBT, permissible HLA MMs are based on the concept of fetomaternal tolerance.

1.4 Foetal development and tolerance towards NIMA

In human pregnancy, exposure and interaction between the maternal (decidua) and foetal (trophoblast) tissues results in trans-placental cell traffic (Erlebacher 2013). The presence of maternal cells in the foetus is termed maternal microchimaerism (MMc) and the presence of foetal cells in the mother is termed foetal microchimaerism. Both the mother and foetus are therefore exposed to each other's semi-allogeneic molecules and for the pregnancy to be a success, must avoid immunological rejection of one another. Significant attention has focused on maternal acceptance of the semi-allogeneic foetus but far fewer studies have focussed on the reciprocal problem of how the foetus is able to tolerate its semi-allogeneic host (Erlebacher 2013, Mold and McCune 2012).

Fetomaternal tolerance was previously hypothesised to be a consequence of an inert, functionally impaired foetal immune response with limited antigen experience and insufficient memory (Burt 2013). The evidence discussed below outlines that this hypothesis is no longer sufficient and rather, that the foetal immune system develops in such a way that it learns to tolerate non-self and specifically, the NIMA and that this can be exploited in the HLA mismatched transplantation setting.

1.4.1 Pathways of foetal allorecognition of its mother

There are three main mechanisms of allorecognition between the graft and host and these are described below in the context of the foetal host and the maternal microchimaeric cells expressing NIMA as the graft **(Figure 1.7)**. The direct pathway involves the recognition of intact class I or class II NIMA molecules

expressed on maternal cells by foetal T$_H$ and CTL clones that express TCRs specific for allogeneic NIMA HLA-peptide complexes. Direct allorecognition is mainly thought to be due to the cross reactivity of a host TCR (specific for self-HLA and a viral or bacterial peptide) with a non-self HLA due to TCR interaction outside the peptide-binding groove, regardless of the peptide present (Bracamonte-Baran and Burlingham 2015). The indirect pathway involves the phagocytosis of microchimaeric maternal cells by foetal APCs, which process soluble NIMA HLA and results in the expression of foetal HLA class II molecules with specific allogeneic NIMA-derived peptides bound on the surface of foetal APCs. Foetal T$_H$ clones recognise the NIMA peptide-self HLA class II complex and elicit an immune response.

Figure 1.7 Pathways of allorecognition. In the context of pregnancy, the role of donor cells in the foetus is played by maternal cells expressing NIMA (red). Direct pathway implies the recognition of intact allo-HLA molecules and indirect pathway implies the recognition of allopeptide–self HLA II complexes. In the semi-direct pathway, allogeneic HLA molecules (NIMA) are acquired via exosomes/trogocytosis. IMA are shown in grey, NIMA in red, and IPA in blue. (Bracamonte-Baran and Burlingham 2015).
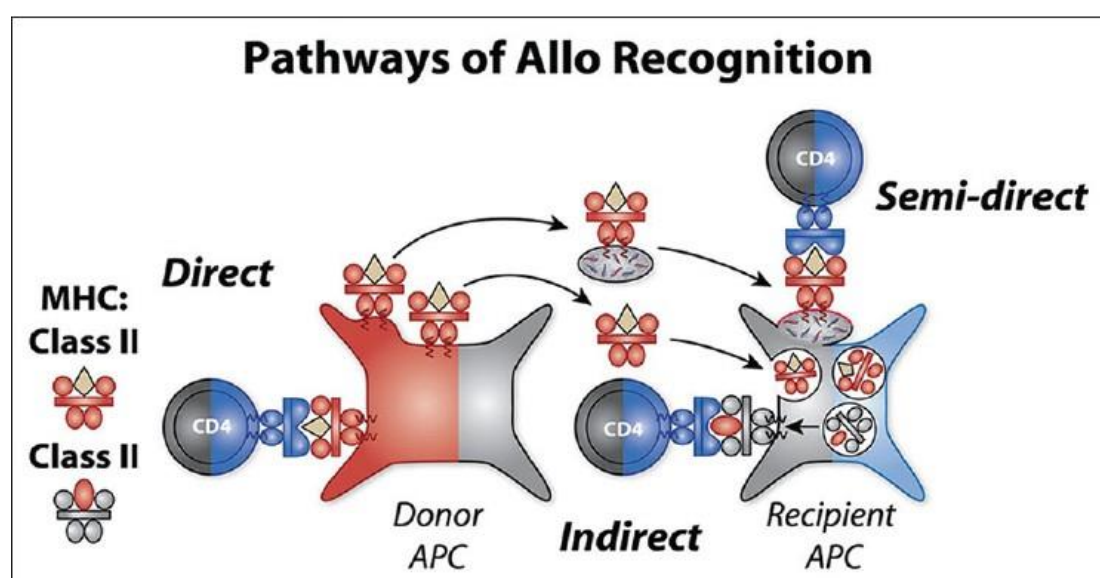
T$_H$ cells activated through the indirect pathway are able to amplify or regulate CTL cells that have been activated through direct allorecognition (Marino, *et al* 2016b). This cross-talk relies on a 'three-cell' model, whereby both T$_H$ and CTLs are activated by the same APC. However, immune responses in transplantation appear to contradict this model because CTL and T$_H$ cells are activated through different APCs (donor/graft and recipient/host, respectively) and thus amplification appears to necessitate a fourth cell. A semi-direct pathway of allorecognition has more recently been proposed to solve this paradox (Marino, *et al* 2016b). In this pathway, maternal cells deliver exosomes containing allogeneic NIMA HLA molecules, which are acquired by a specific subset of foetal DCs. Foetal DCs expressing NIMA HLA molecules are then able to interact with maternal T cells in a direct-like fashion as well as through the indirect pathway. This process has recently been shown to be critical in direct pathway acute rejection of allografts (Liu, *et al* 2016, Marino, *et al* 2016a).

DCs also have an additional pathway, known as cross-presentation (Joffre, *et al* 2012). This involves the uptake and proteasome mediated-processing of extracellular antigens. The resulting peptides are presented on DCs by self-HLA class I molecules for recognition by CD8$^+$ CTLs. During an immune response to an infection, cross-presentation permits the priming of naive CD8$^+$ CTLs without the need for the APC to be infected by a virus (Joffre, *et al* 2012).

Maternal T cells, B cells, DCs and macrophages have been detected in the PB and organs of the foetus, including the lymphoid organs (Drabbels, *et al* 2011). The developing foetus is therefore not antigen inexperienced (Dierselhuis, *et al* 2012, Dierselhuis, *et al* 2014, Vernochet, *et al* 2005) and each of the pathways of

allorecognition are possible. Subsequent effector responses towards the semi-allogeneic mother therefore need to be either prevented, or far more likely given the requirement for the development of a normal, healthy immune response; controlled, such as through the development of immunological tolerance.

1.4.2 Immunological tolerance

1.4.2.1 Discovery of fetomaternal tolerance

Seventy years ago, Owen made the remarkable discovery that most dizygotic twin cattle were born with a stable mixture of each other's erythrocytes and that this chimaerism persisted into adulthood (Owen 1945). These cattle were later shown to be able to accept post-natal transplantation of each-others skin grafts (Billingham, *et al* 1952). These findings and the seminal experiments by Billingham, Brent and Medawar defined the principles underpinning the immunological basis of tolerance (Billingham, *et al* 1953).

Owen made the observation that some Rh-negative women did not develop anti-Rh antibodies during the pregnancy of a Rh-positive foetus if the mothers of the pregnant women were Rh-positive (Owen, *et al* 1954). This suggests the pregnant women had acquired tolerance towards the NIMA, which in this case was Rh. This phenomenon is referred to as fetomaternal tolerance. Broadly, there are two types of tolerance: central and peripheral.

1.4.2.2 Central tolerance

Central tolerance is the mechanism by which T and B lymphocytes are rendered non-reactive to self. Maturing lymphocytes are exposed to self-antigens in the

thymus (T cells) or BM (B cells) and auto-reactive clones are deleted before they develop into immunocompetent cells.

During the early stages of T lymphocyte development, cells randomly generate unique clonal TCRs through somatic V(D)J recombination and gene conversion (which also occurs for the B cell receptor during B cell development). CD4⁻CD8⁻ T cell precursors whose rearranged β-chain cannot associate with a rearranged α-chain at the cell surface die by apoptosis. Those with successful associations receive survival signals, gain CD4⁺CD8⁺ expression and subsequently undergo thymic selection. Survival depends upon the interaction of the TCR with self-peptide-HLA complexes. In a series of elegant experiments by Doherty and Zinkernagel, T cells were shown to be MHC restricted, that is, if the TCR cannot recognise self-peptide-MHC there is death by neglect (Doherty and Zinkernagel, 1975) . Too much signalling (i.e. risk of autoimmunity) promotes acute apoptosis (negative selection). An intermediate level of TCR signalling initiates effective maturation (positive selection) and those expressing TCRs that bind self-peptide-MHC class I complexes become CD8⁺ T cells, whereas those that bind self-peptide-MHC class II complexes become CD4⁺ T cells.

Deletion of T (and B) cell precursors reactive against NIMA during negative selection under the presence of MMc in the foetal thymus (and BM) (Srivatsa, *et al* 2003, Vernochet, *et al* 2005) may be one mechanism of tolerance towards NIMA. However, fetomaternal tolerance cannot be explained by clonal deletion alone because foetal CD4⁺ and CD8⁺ T cells are able to elicit an immune response against NIMA *in vitro* and therefore must have escaped clonal deletion (Hadley,

et al 1990, Jankowska-Gan*, et al* 2012, Mold*, et al* 2008, Roelen*, et al* 1995). To deal with this, there is a second line of response, known as peripheral tolerance.

1.4.2.3 Peripheral tolerance

Peripheral tolerance occurs after lymphocyte development in the peripheral tissues and lymph nodes (LN). Mechanisms of peripheral tolerance include immune privilege, anergy and regulation.

Immune privilege refers to the ability of a tissue to actively regulate and direct immune responses that take place in its "territory." The pregnancy environment may act as an immune privileged site, given that amniotic fluid is rich in the immunomodulatory cytokine, transforming growth factor beta (TGF-β), which can modulate DCs in such a way that they are unable to give a full stimulus to T cells (Erlebacher 2013, Guleria and Sayegh 2007). If there is not appropriate co-stimulation or if the ligand for the TCR is of insufficient affinity to initiate the full spectrum of responses, the T cell is unresponsive upon TCR engagement, termed anergy. Anergic cells are unable to proliferate or secrete cytokines upon TCR mediated re-stimulation (Erlebacher 2013, Guleria and Sayegh 2007).

The primary mechanism of peripheral tolerance involves a specialised population of T lymphocytes known as regulatory T cells (Tregs). The existence of Tregs was first proposed to explain the observation that peripheral tolerance towards foreign antigens could be induced in the absence of a functional thymus (Gershon and Kondo 1970) and were later identified as a subpopulation of CD4^{+} T cells that could prevent autoimmune disease (Sakaguchi*, et al* 1985). Multiple populations of Tregs have been characterised but the best known and most

extensively studied are CD4^{+} cells that express the high affinity interleukin (IL)-2 receptor (CD25) and the transcription factor FOXP3. These natural Tregs become committed to a regulatory fate during thymic development, when they are identified as potentially self-reactive through high affinity binding to self-peptide-HLA complexes. In the periphery, Tregs can also develop from conventional T cells under certain conditions (including strength of the antigen signal, the presence of TGF-β and IL-10 and other signals from DCs) and are termed peripheral or induced Tregs.

Multiple Treg populations are likely to be involved in controlling response towards NIMA. CD4^{+}CD25^{+}FOXP3^{+} Tregs have been implicated in the suppression of anti-NIMA responses in humans and were proposed to have been induced in response to MMc in the LN rather than during thymic development (Mold, *et al* 2008). However, in mice, a populaton of regulatory cells positive for TGF-β and LAP but not FOXP3, have been associated with a decreased response towards NIMA and these cells were proposed to be T$_H$3 regulatory cells, thought to mediate mucosal tolerance (Molitor-Dart, *et al* 2007). These cells could arise after exposure to NIMA from drinking amniotic fluid containing soluble NIMA and TGF-β or from breastfeeding (Andrassy, *et al* 2003, Campbell, *et al* 1984).

Mechanisms of Treg mediated suppression are diverse and seem to be dependent on the context of their activation and the environment in which they are operating. Tregs generally act in a contact-dependent fashion and by secreting IL-10 and TGF-β, which inhibit T cell proliferation (Shevach 2011). Tregs have been shown to suppress proliferative responses against NIMA via the production of TGF-β (Molitor-Dart, *et al* 2007, Mold, *et al* 2008). Other methods of suppression

include diminishing conventional T cell activation or function by limiting growth factors such as IL-2, cytotoxicity towards a target cell or by modulation of DC function (Shevach 2011).

Regulatory responses towards NIMA may be promoted by the timing of exposure. The human foetal immune system has been shown to be enriched for Tregs at early stages during development, which decrease in frequency during the third gestation (Cupedo, *et al* 2005, Darrasse-Jèze, *et al* 2006, Darrasse-Jèze, *et al* 2005, Michaëlsson, *et al* 2006). These changes are hypothesised to occur because the human adaptive immune system is built from layers of haematopoiesis that differ in immunological function and occur at different stages of development (Mold, *et al* 2010). In an elegant study by Mold and colleagues, both foetal and adult T cells were shown to be highly proliferative in response to alloantigen *in vitro* but foetal CD4^{+} T cells expressed different gene signatures and were more likely to produce Tregs when stimulated with allogeneic PB cells in a mixed lymphocyte reaction (MLR) (Mold, *et al* 2010). Molecular and functional differences between foetal and adult HSCs were also identified and the authors speculated that foetal and adult CD4^{+} T cells were derived from two different pools of HSCs. The authors hypothesised that the foetal immune system is biased towards a state of tolerance and that a shift towards immune reactivity occurs during the transition to an adult immune system.

Tolerance towards NIMA appears in some cases to last beyond foetal development. Lymphocytes of children have been shown to display a Treg mediated suppression of proliferation against maternal but not paternal cells, possibly due to long-lived memory T cells (Mold, *et al* 2008, Moretta, *et al* 1999,

Tsafrir, *et al* 2000, Zhang and Miller 1993). Alternatively, new Tregs could have been generated from repeated exposure to NIMA through the persistence of MMc throughout childhood, which maintained NIMA-specific tolerance. HSCs of maternal origin have been detected in the BM of mice (Dutta, *et al* 2010, Dutta, *et al* 2009) and in humans, MMc has been detected decades after birth (Loubiere, *et al* 2006), which suggests the transfer of multipotent/pluripotent stem cells during pregnancy which are able to continuously divide and replenish the pool of MMc. Long-lasting tolerance towards NIMA has immunological implications to both related and unrelated transplantation.

1.4.3   Clinical studies of NIMA matching in transplantation

In haploidentical related donor transplantation, the mismatched haplotype can be either the NIMA or NIPA. For example, in **Figure 1.8**, the patient has inherited the paternal haplotype "A" (A1-B8-DR17) and maternal haplotype "C" (A3-B7-DR15). Sibling 1 is an haploidentical match to the patient because they both share the paternal "A" haplotype. The mismatched haplotype between the patient and sibling 1, from the host-versus-graft (HvG) perspective, is the sibling 1 maternal haplotype "D" (A24-B57-DR7). This mismatched haplotype D of sibling 1 is matched to the patient NIMA and thus the transplant is termed a "NIMA match" (NIMA+) in the HvG direction. NIMA matching in the HvG direction is considered when evaluating the influence of NIMA in renal transplantation. In a similar manner, sibling 2 is a "NIPA match" (NIPA+) to the patient in the HvG direction for the "B" haplotype.

In haploidentical sibling HSCT, NIMA matching refers to the mismatched haplotype in the GvH direction i.e. the NIMA matched haplotype is the patient mismatched haplotype. In **Figure 1.8**, sibling 1 is a NIMA match to the patient because the mismatched patient "C" haplotype is matched to the sibling NIMA. In a similar manner, sibling 2 is a NIPA matched to the patient because the mismatched patient "A" haplotype is matched to the sibling 2 NIPA.

Tolerance towards NIMA has been observed in the clinical setting of related and unrelated donor transplantation for both solid organ and HSC grafts.

Figure 1.8 HLA matching status between a patient and their siblings. The patient has inherited haplotype "A" (IPA) and "C" (IMA) from their father and mother, respectively. Sibling 1 is a haploidentical match to the patient because they have both share the paternal "A" haplotype. The mismatched haplotype between the patient and sibling 1, from the HvG perspective, is the sibling 1 haplotype "D" but this is a match to the patient NIMA haplotype and termed a "NIMA match" in the GvH direction. Sibling 1 is also NIMA matched to the patient in the GvH direction because the mismatched patient "C" haplotype is matched to the sibling NIMA. Similarly, the patient and sibling 2 are NIPA matched in both the HvG ("B") and GvH ("A") directions.
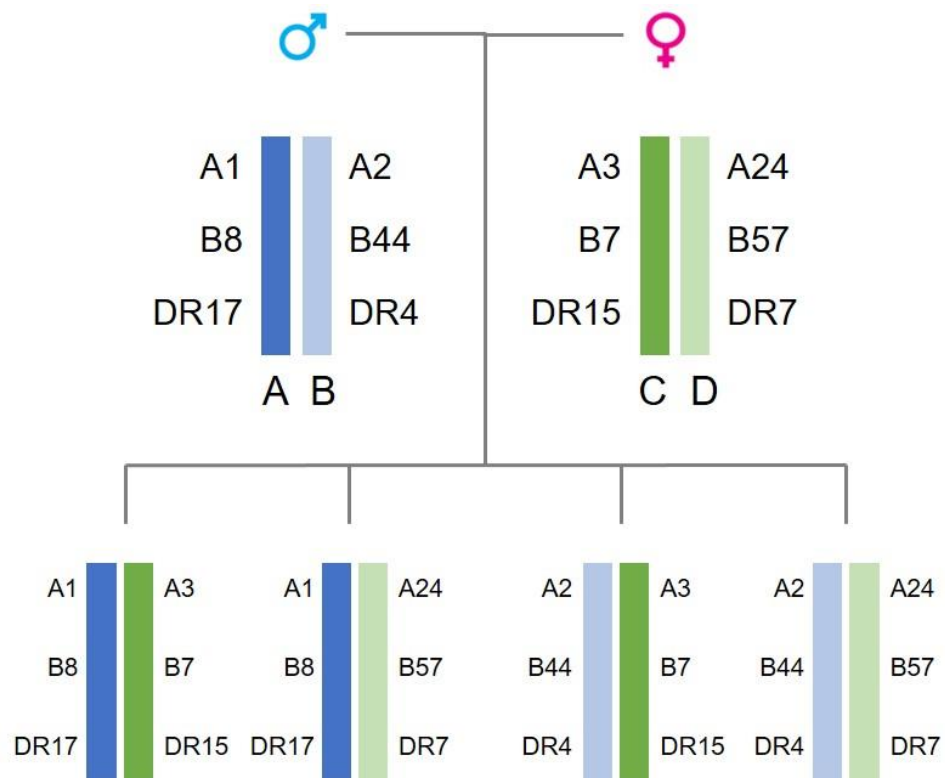
1.4.3.1 Renal transplantation

In a multi-centre study, Burlingham and colleagues reported a significant benefit to ten-year renal graft survival for NIMA+ grafts (77%) compared to NIPA+ grafts (49%) (Burlingham, *et al* 1998). Survival of NIMA+ grafts paralleled that of HLA identical grafts. The NIMA effect was especially apparent when patients were not taking cyclosporine, giving an early clinical indication that the NIMA effect may be partially dependent on a T cell response. Furthermore, the NIMA effect has also been reported in a retrospective study of over 600 patients that received an (unrelated) deceased donor renal transplant (Smits, *et al* 1998). Single HLA-A antigen mismatched renal grafts whereby the HLA-A MM was a NIMA+ were associated with a graft survival that was actually superior to that of zero mismatched grafts. This suggested that the NIMA effect was caused by an active process that down-regulated the allogeneic immune response, rather than a clonal deletion of NIMA reactive cells that caused a repertoire devoid of cells capable of an effector response towards NIMA alloantigens.

In contrast to NIMA+ sibling grafts, maternal renal grafts have repeatedly not been associated with a graft survival advantage compared to paternal grafts and in some cases, maternal grafts have a poorer prognosis (Lim, *et al* 2016, Opelz 1990, Panajotopoulos, *et al* 1990). This includes data from The Collaborative Transplant Study of over 5,000 parent-to-child renal transplants (Opelz 1990). In a more recent Australian retrospective analysis of 1,139 renal transplant recipients, maternal grafts were associated with greater than a 1.5-fold higher risk of early and multiple rejection episodes, with more than a 30% higher risk of graft loss (Lim, *et al* 2016). This analysis was particularly important because it

was the first study comparing maternal and paternal renal grafts in the era of modern immunosuppression and had a median follow-up time of seven years.

This lack of a tolerogenic NIMA effect in maternal donor renal transplantation is known as the "NIMA paradox." The association between maternal grafts and rejection appeared to be more apparent in adult recipients compared with younger recipients, suggesting an age-related immunological response towards NIMA (Lim, *et al* 2016). This parallels conflicting earlier MLR *in vitro* studies that failed to identify a decreased direct-pathway alloreactivity towards maternal versus paternal cells in adults and contrasts studies of fetomaternal tolerance (Hadley, *et al* 1990, Jankowska-Gan, *et al* 2012, Mold, *et al* 2008, Roelen, *et al* 1995, van den Boogaardt, *et al* 2005).

The poor survival of maternal renal grafts may relate to passenger lymphocytes in the renal graft that elicit an immune response in the recipient. The type of immune response may differ depending on the antigens that these passenger lymphocytes have encountered and at what time point during the donor's life. In maternal donor renal transplantation, maternal passenger lymphocytes would have previously been exposed to the mismatched antigens of her offspring (the IPA) during a pregnancy that occurred in adult life and it is well established that mothers can become sensitised to foetal IPAs (Claas, *et al* 1988). In contrast, in sibling donor transplantation, donor sibling passenger lymphocytes will have also been previously exposed to the recipient mismatched antigens but crucially, exposure would have occurred during foetal development from MMc. The recipient mismatched antigens are also a NIMA+ and therefore in addition to there being a HvG NIMA+, there is also a GvH NIMA+ mismatch. In the example

given earlier in **Figure 1.8**, if sibling 1 is the renal graft donor, passenger lymphocytes in the graft will recognise the recipient haplotype "C" IMA as non-self but these recipient IMA are actually the NIMA of the donor passenger lymphocytes.

Evidence that it is not just the recipient's immune status that matters in renal transplantation and that the *donor immune status* may also contribute to post-transplant outcome in what is termed "bi-directional alloreactivity" has come from a recent study by Jankowska-Gan and colleagues (Jankowska-Gan, *et al* 2012). Immune regulation in both the recipient anti-donor (HvG) and donor anti-recipient (GvH) directions were analysed in 29 renal transplant pairs using a trans-vivo Delayed Type Hypersensitivity assay. The pre-transplant recipient response to the donor alone did not predict transplant outcome. However, the inclusion of the pre-transplant donor immune response to the recipient, together with the pre-transplant recipient response to the donor, identified in 9/18 patients, was significantly associated with less rejection. Seven of the nine transplants with only unidirectional or no pre-transplant immune regulation experienced acute rejection. The assay measured regulation via the indirect pathway and therefore both donor tissue APC and passenger lymphocyte T cells were implicated as critical players in determining allograft outcome.

1.4.3.2 Haploidentical HSCT

Studies have also demonstrated a beneficial effect of NIMA matching in haploidentical sibling HSCT for patients with malignant disease, during an era when haploidentical transplants were very high risk (van Rood, *et al* 2002).

Historically, haploidentical sibling donor HSCT was performed with T cell depletion (TCD) or positive selection of CD34+ cells to overcome complications from severe aGvHD and these methods were associated with graft failure, fatal opportunistic infections and relapse. Early studies demonstrated that T cell replete haploidentical HSCT was possible by exploiting fetomaternal tolerance and NIMA+ sibling HSCT was proposed as an alternative to *in vivo* or *ex vivo* TCD (Ichinohe*, et al* 2004). In a 2002 retrospective analysis, clinical outcomes of non-TCD BMT were compared in 269 recipients that received either an haploidentical graft from a parent or a NIMA+ or NIPA+ sibling and primarily a cyclosporine based GvHD prophylaxis (van Rood*, et al* 2002). NIMA matched sibling grafts were associated with a lower incidence of acute II-IV GvHD and improved survival compared to NIPA matched sibling grafts and parental grafts. Maternal grafts were associated with a lower incidence of cGvHD and TRM compared to paternal grafts.

The lower incidence of aGvHD using NIMA+ sibling donors in non-TCD BMT has also been confirmed in a Japanese study of 35 patients with advanced malignant disease that received a PB sibling or maternal graft and a tacrolimus based GvHD prophylaxis (Ichinohe*, et al* 2004). All of these cases were selected on the basis of chimaerism present in the donor that was specific to the recipient mismatched antigens and assumed to be of maternal origin (sibling donors) or foetal origin (maternal donors). However, 10% of patients that received a NIMA+ graft still experienced severe aGvHD, despite the presence of MMc, demonstrating that the presence of MMc does not always correlate to a tolerogenic NIMA effect (Ichinohe*, et al* 2004) and highlighted a clinical need to predict the risk of GvHD

in NIMA+ HSCT. In a follow-up study, 16 Japanese patients were analysed for late complications, including the severity of cGvHD, requirement for immunosuppression and status of primary disease (Kanda, *et al* 2009). The authors identified that long-term survival without continuous immunosuppression is possible after non-TCD NIMA+ haploidentical HSCT using a MMc positive donor, despite the frequent occurrence of moderate-to-severe cGvHD.

The significant improvements in clinical outcome associated with the recent development of new T cell replete protocols has led to an increase in the use of haploidentical donors (Chang, *et al* 2016). There has only been one study of NIMA matching in T cell replete haploidentical HSCT in the modern era. Wang and colleagues extensively analysed the outcomes of 1,256 haploidentical HSCT recipients with malignant disease that were treated at Peking University Institute of Haematology (Wang, *et al* 2014). NIMA+ sibling donors were associated with a lower incidence of aGvHD compared with parental and NIPA+ sibling donors. There was no significant impact of NIMA matching on non-relapse mortality, cGvHD, relapse or survival. Maternal donors were associated with a higher incidence of aGvHD compared to paternal donors, similar to the reported poor outcomes of maternal renal grafts compared to paternal grafts (Lim, *et al* 2016, Panajotopoulos, *et al* 1990). Poorer clinical outcomes (GvHD, non-relapse mortality and survival) of haploidentical HSCT were also observed for female donors and older donors (Wang, *et al* 2014). The authors concluded younger, male, NIMA+ donors should be the preferred donor choice for T cell replete haploidentical HSCT and that older mothers and NIPA+ donors should probably

be avoided. Given these findings and the renewed interest in haploidentical HSCT, a European study that compares the outcomes of different types of haploidentical donors, to identify if there was still a NIMA effect present, would be incredibly worthwhile.

1.4.3.3 Unrelated CBT

A possible extension to the observed NIMA effect in related haploidentical BMT is the setting of unrelated CBT because of the ability to determine the CB NIMA from maternal HLA typing. Re-exposure to NIMA can occur in CBT if the MM between the CB and recipient is a match for the donor NIMA. van Rood and colleagues retrospectively evaluated the influence of NIMA matching on outcomes after CBT using data on 1,121 patients with malignant disease that received a graft from the New York Cord Bank (NYCB) (van Rood, *et al* 2009). NIMA matching status was evaluated after the transplants were performed and of the 1,059 HLA mismatched grafts, 79 (7%) were NIMA+ at *HLA-A*, *-B* or *–DRB1* by chance. NIMA+ CB grafts were associated with a faster time to neutrophil engraftment, especially for recipients of a poor cell dose. TRM was also lower after NIMA+ CBT and this was especially apparent in patients younger than 10 years old. These benefits translated to improved survival after NIMA+ CBT compared to NIMA-, HLA mismatched CBT. Intriguingly, despite the lack of an increase in GvHD, NIMA+ CB grafts also showed a trend towards a lower risk of relapse (van Rood, *et al* 2009). A subsequent multicentre case-control retrospective analysis by the Eurocord-European Blood and Marrow Transplant Group (EBMT) and the Centre for International Blood and Marrow Transplant Research (CIBMTR) confirmed the survival advantage in NIMA+ CBT (Rocha, *et*

al 2012). The five-year incidence of TRM in recipients of NIMA+ grafts was 18% compared to 32% in recipients of NIMA- mismatched grafts. Consequently, the 5-year probability of overall survival (OS) was 55% after NIMA+ CBT compared to 38% after NIMA- mismatched CBT.

In contrast to related haploidentical transplantation, NIMA matching has not been associated with a decrease in aGvHD in CBT (van Rood*, et al* 2009). The authors argued that CBT was not comparable to haploidentical HSCT because of differences in HLA matching. Haploidentical donors share a complete HLA haplotype that is matched to the allelic level whereas CB grafts have only been historically matched to the phenotypic level at *HLA-A* and *-B* and to allele level at *-DRB1*. Similarly, the NIMA is mismatched at allelic level across a complete HLA haplotype between siblings but only at one, or rarely, two antigens at phenotype level in CBT. Furthermore, CB is already associated with a lower incidence of GvHD compared to haploidentical donors, possibly due to its Treg properties, which may have masked any beneficial effect of NIMA matching on GvHD incidence.

Furthermore, foetal-maternal tolerance may benefit CBT recipients with malignant disease, irrelevant of NIMA matching status. It is hypothesised that maternal microchimaeric cells, sensitised against foetal IPA, are present in the CB graft and mediate a GvL response in the recipient (van Rood*, et al* 2012). Recipients that shared one or more HLA antigens (*HLA-A, -B*, or *-DRB1*) with their CB donor's IPAs had a significant decrease in relapse compared with those that did not, without an increase in GvHD (van Rood*, et al* 2012). The mechanism of reduced relapse after IPA matched CBTs was not established and MMc in the CB

graft was not quantified. However, almost all patients received calcineurin inhibitors and the majority also received anti-thymocyte globulin (ATG) and therefore the T or natural killer (NK) cell mediated GvL effect was shown not to be inhibited by either of these.

1.4.4 NIMA matching in murine models of transplantation

The potential tolerogenic role of NIMA has also been clearly demonstrated in mice. The injection of pregnant H-2^d-negative female mice with an H-2^d peptide can result in profound suppression of the offspring's alloreactive CD4$^+$ T cell response (via the indirect pathway) to H-2^d, demonstrating the formation of tolerance from foetal exposure to alloantigen (Andrassy, *et al* 2003). In a model of transplantation, H-2$^{b/d}$ females are bred with H-2$^{b/b}$ males to yield NIMA^d exposed H-2$^{b/b}$ offspring and H-2$^{b/b}$ females are bred with H-2$^{b/d}$ males yield NIPA^d exposed H-2$^{b/b}$ control offspring for comparison studies (Zhang and Miller 1993). NIMA^d skin allografts in H-2$^{b/b}$ offspring exhibit prolonged survival compared to NIPA^d allografts transplanted in H2$^{b/b}$ offspring (Zhang and Miller 1993). Recipients of NIMA^d BM grafts exhibit a lower incidence of GvHD and improved survival compared to recipients of NIPA^d grafts but this NIMA effect was abolished when CD4$^+$CD25$^+$ T cells were depleted from the NIMA^d graft (Matsuoka, *et al* 2006). The NIMA effect does not appear to be due to the inability to mount an effector response. In a heart allograft model, both NIMA^d exposed and control mice are capable of producing an allospecific T effector response (production of interferon-gamma (IFN-γ) and IL-2) but this is reduced in NIMA^d mice (Molitor-Dart, *et al* 2007). NIMA^d transplants are also associated with an increase in Tregs within the LN and heart allograft, which also produced an

abundance of TGF-β and IL-10 (Molitor-Dart, *et al* 2007). These NIMA[d] heart allografts remarkably survived for 180 days or more in 57% of cases, without any additional drugs or conditioning, compared to rejection of NIPA[d] allografts within 11 days in control experiments (Molitor-Dart, *et al* 2007). These experiments demonstrate tolerance towards NIMA is a consequence of foetal exposure to NIMA during pregnancy and that this tolerance appears to be mediated by Tregs.

However, NIMA-specific tolerance is not consistent between different genders and strains. NIMA[d] heart allografts show a 57% graft survival in male recipients (Andrassy, *et al* 2003) but just 25% graft survival in female recipients, albeit NIMA[d] graft survival in females is still higher than the NIPA[d] control (Molitor-Dart, *et al* 2008). Female mice were speculated to exhibit weaker tolerance due to oestrogen-mediated enhancement of an effector response, which could outweigh any regulatory response. Furthermore, tolerance was only observed if the NIMA was H-2[d] and sensitisation occurred if the NIMA was H-2[k] or H-2[b], which may be due to differences in the timings of immune system development between the different strains (Molitor-Dart, *et al* 2008).

1.4.5 Maternal microchimaerism and a 'split tolerance' towards NIMA

Tolerance towards NIMA+ grafts can occur despite the presence of functional NIMA specific alloreactivity (Opelz 1990, Ichinohe, *et al* 2004, Molitor-Dart, *et al* 2007, Molitor-Dart, *et al* 2008, Kanda, *et al* 2009, Araki, *et al* 2010, Lim, *et al* 2016). In a murine model, it has been possible to identify and distinguish between two separate groups: those that are tolerant towards NIMA and those that are sensitised towards NIMA, based on the release of IFN-γ in an MLR-ELISPOT assay

(Araki*, et al* 2010). Those that were tolerant had higher levels of MMc and produced significantly less IFN-γ when stimulated with NIMA. This raises the possibility that *in vitro* assays that measure IFN-γ production in response to stimulation against NIMA could have a clinical role in predicting the outcome of NIMA+ transplants.

Furthermore, studies have failed to demonstrate reduced alloreactivity towards NIMA in adults through MLRs that measure direct pathway responses (Hadley, *et al* 1990, Roelen, *et al* 1995, Akiyama, *et al* 2011), yet others have shown a Treg mediated tolerance associated with indirect allorecognition (Akiyama*, et al* 2011, Jankowska-Gan*, et al* 2012, van Halteren*, et al* 2009). Taken together, this suggests the existence of a "split tolerance" phenomenon associated with the NIMA effect, in which regulation to a NIMA-specific indirect pathway is induced without tolerogenic impact on the direct pathway (Bracamonte-Baran and Burlingham 2015).

How such a small proportion of maternal microchimaeric cells can lead to profound tolerance in transplantation, especially in the presence of "split tolerance," remains unclear. The semi-direct pathway of allorecognition may provide a mechanistic link (Bracamonte-Baran and Burlingham 2015). Maternal microchimaeric monocytes, macrophages and DCs have been identified in mice (CD11c and CD11b subsets in flow cytometry analyses) and these can release exosomes containing intact NIMA MHC that can be acquired by host DC in a phenomenon known as "cross-dressing" (Campana*, et al* 2015). APCs dimly expressing allogeneic intact NIMA MHC have been detected in mice that are tolerant of NIMA+ grafts but this APC population is more transient in rejecters of

NIMA+ grafts (Dutta, *et al* 2009, Molitor-Dart, *et al* 2007). APC acquisition of intact NIMA MHC may require a certain threshold and/or quality of MMc and this may explain why low levels of MMc are associated with sensitisation towards NIMA (Andrassy, *et al* 2003, Bracamonte-Baran, *et al* 2017, Molitor-Dart, *et al* 2008, Molitor-Dart, *et al* 2007). An elegant study exploring the mechanisms behind this phenomenon and a proposed model of split-tolerance, shown in **Figure 1.9**, has recently been published (Bracamonte-Baran, *et al* 2017). Membrane alloantigen acquisition (m-AAQ) by DCs was shown to occur in some but not all mice and positively correlated with MMc levels. In those positive for m-AAQ, a split tolerance was observed, where the direct pathway of allorecognition remained intact but stimulation through the indirect pathway of allorecognition resulted in anergy (shown to be through T cell cycle arrest after one round of cell division). A heart allograft model paralleled previous observations, where in m-AAQ+ mice, an acute rejection pathway was present but if mice survived this effector response, they remained free from chronic rejection and exhibited prolonged survival (Bracamonte-Baran, *et al* 2017, Burlingham, *et al* 1998). In contrast, m-AAQ- mice exhibited alloreactivity through the direct and indirect pathways and succumbed to acute rejection.

Figure 1.9 Proposed models for microchimaerism and exosome-mediated split tolerance. Models of DC nonclassical (cross-dressing) and classical MHC-II pathways of allopresentation in mice having non-mAAQ (A) and mAAQ+ (B) forms of microchimaerism. (A) In a non-mAAQ condition, MMc-derived soluble allo-MHC molecules (red) are classically processed and presented in a self–MHC-II–restricted manner, leading to active indirect pathway (TEa) cells, whereas scarcity of intact allo-MHCs makes direct recognition (4C) inactive. The exosomes are all derived from self-tissue–resident DC, with ample CD86 and low PD-L1 coexpression, reinforcing self-MHC–restricted alloreactivity (* indicates a membrane fusion process that may occur on contact or only after exosome internalization, with recycling of the fused patch to the cell surface). (B) In mAAQ+ settings, not only are soluble allo-MHCs released from MMc, but also exosomes, generating a "Janus-faced" DC. On the left, nonclassical side, exosome-acquired intact allo-MHCs are colocalized with acquired CD86 in microdomains that exclude PD-L1, leading to activation of direct T-cell clones (4C). On the right, exosome acquisition leads to reprogramming of the classical MHC II pathway ("?" indicates that exosome-associated miRNA effects on PD-L1 mRNA translation are postulated), such that PD-L1 is present in the microdomains expressing the allopeptide/self–MHC-II complexes, generating abortive activation/anergy of indirect T-cell clones (TEa; red X) via PD-1 and TcR microclustering (Bracamonte-Baran, *et al* 2017).
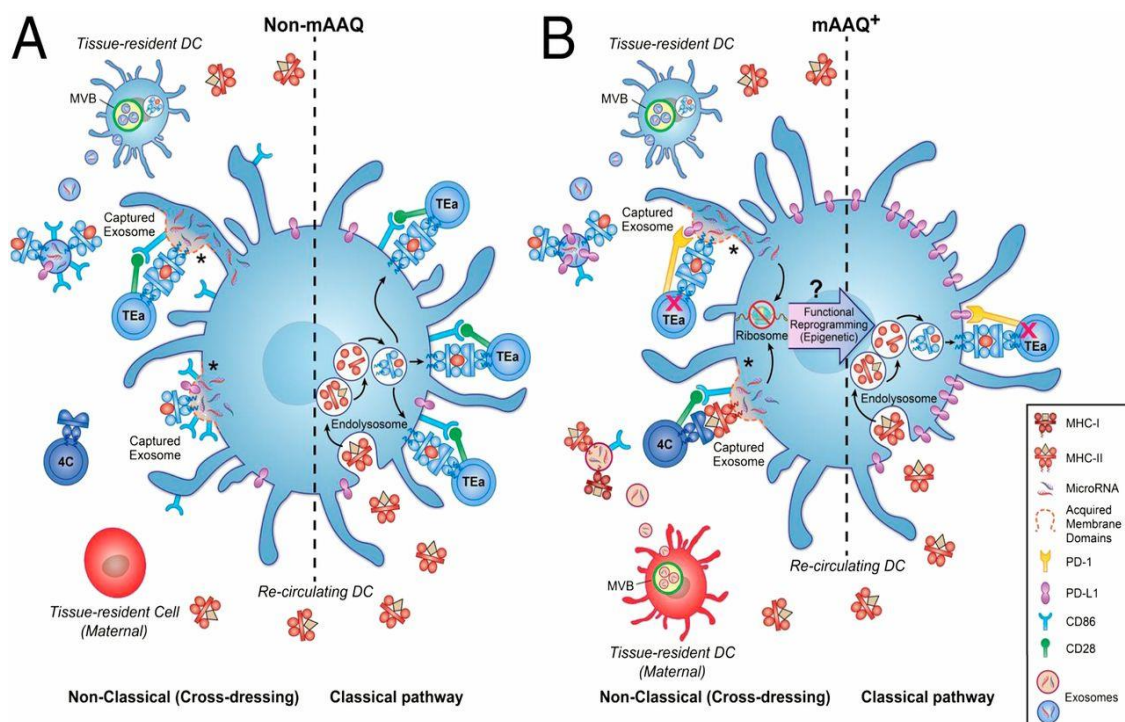
The differences in stimulation by these two pathways of allorecognition acting via the same DC in m-AAQ+ mice appeared to be due to differences in the microdomain environments where the different T cell interactions were taking place (Bracamonte-Baran, *et al* 2017). DC microdomains with intact acquired MHC alloantigen (and the serum enriched for extracellular vesicle fractions) were enriched for CD86, a ligand that provides the co-stimulatory signal necessary for T cell activation and survival. The immunomodulator, PD-L1 was absent from these microdomains. In contrast, PD-L1 was localised to microdomains containing allopeptide+self-MHC, which binds to PD-1 on T cells and delivers a signal that inhibits TCR-mediated activation and proliferation. It therefore seems the lack of proliferation of T cells stimulated via the indirect pathway was due to the presence of PD-L1 and indeed, if PD-L1 was blocked in the cultures, T cells were able to proliferate in response to allopeptide+self-MHC. Given that increased expression of PD-L1 was observed in these m-AAQ+ mice but was absent from the vesicles themselves, the authors hypothesised that vesicle microRNA had somehow reprogrammed the DC to upregulate endogenous expression of PD-L1.

The biological reasons for a naturally occurring split-tolerance towards NIMA are unclear. However, some speculation may be made based on the recent finding that in mice, MMc-induced Treg development impacts cross-generational reproductive fitness through NIMA-specific Tregs in female offspring (Kinder, *et al* 2015). This is in agreement with the observation that increased levels of MMc in adult women are associated with a reduced rate of preeclampsia and recurrent miscarriage (associated with aberrant foetal tolerance), in what is termed the

grandmother effect (Gammill, *et al* 2011, Gammill, *et al* 2015). Although such tolerance appears to protect against foetal loss, an acute inflammatory response is however, required to facilitate embryo implantation and the presence of a split-tolerance to NIMA may be the best approach to facilitate both (Bracamonte-Baran, *et al* 2017, Kinder, *et al* 2015). This may explain why MMc has been associated with autoimmune diseases such as scleroderma (Lambert, *et al* 2004). Furthermore, split-tolerance towards NIMA may also explain why Rh-negative women exposed to Rh from their mothers, did not go on to develop anti-Rh antibodies (dependent on the classical pathway of peptide-MHC-II and processing and presentation to T_H cells) during pregnancy of a Rh-positive foetus and yet this did not prevent haemolytic disease of the new-born (Owen, *et al* 1954).

In summary, the foetus develops immunological tolerance towards NIMA, likely though the induction of NIMA specific Tregs. The foetus retains the capability of NIMA specific alloreactivity and overall tolerance appears to be dependent on levels and/or type of MMc, possibly mediated through the semi-direct pathway. This tolerance can be exploited in the setting of HLA mismatched transplantation.

1.5 Aims of this thesis

The central aim of this thesis is to explore the role of NIMA matching in unrelated CBT, with regards to donor availability and clinical outcome and to also develop a sensitive method to quantify the levels of MMc present in CBUs. The main sub aims of this thesis are as follows:

1. To define the *HLA-A*, *-B* and *-DRB1* NIMA of banked CBUs with maternal HLA types available, to generate virtual phenotypes derived from the NIMA and to evaluate whether these increase the number and diversity of HLA phenotypes available for donor searches.

2. To determine whether the inclusion of these virtual phenotypes can provide virtual full matches that can improve donor availability for patients of various ethnicities requiring CBT.

3. To retrospectively determine whether NIMA matching for *HLA-A*, *-B*, *-C* and *–DRB1* had any effect on clinical outcomes after single CBT in recipients with malignant and non-malignant disease.

4. To develop a sensitive q-PCR assay for the detection and quantification of MMc in CB, to determine the levels of MMc in transplanted CBUs and to investigate variables associated with its presence and/or absence.

Chapter 2 Materials and methods

2.1 Stock solutions and commercial kits

Agarose gel (1%)

> 200mL x1 Tris Borate EDTA (TBE) Buffer [Sigma Aldrich]
>
> 2g Agarose Powder [Helena Bioscience]
>
> 20μL GelRed [Biotium]

Agencourt AMPure XP [Beckman Coulter]

Ethanol (>99.8%) [Sigma Aldrich]

Ethanol, 70%

> Ethanol (>99.8%) [Sigma Aldrich] was mixed with de-ionised water in a ratio
>
> of 7:3

Illustra ExoProStar [GE Healthcare Life Sciences]

Long Range PCR kit [Qiagen]

Nuclease free H_2O [Ambion]

Performa DTR plates [EdgeBio]

QiaAmp DNA Maxi kit [Qiagen]

QiAmp DNA Mini Kit [Qiagen]

Sequence Based Typing (SBT) Excellerator kits (*HLA-A, -B, -C, -DRB1*) [GenDx]

SYBR Select Master Mix [Life Technologies]

2.2 Equipment

3130 sixteen capillary genetic analyser [Applied Biosystems]

MicroAmp optical 96-well reaction plate [Applied Biosystems]

MicroAmp optical adhesive film [Applied Biosystems]

FrameStar semi-skirted 96 well plate [4titute]

Nanodrop [Applied Biosystems]

PCR seal [4titude]

Quant Studio 7 Flex Real-Time PCR System [Thermofisher]

SBTengine [Gendx]

Veriti thermal cycler [Applied Biosystems]

2.3 Definitions and terminology

Terminology frequently used throughout this thesis are defined in **Table 2.1**.

Table 2.1 Definitions of HLA typing resolution

Resolution	Definition	Example(s)
Serological	HLA antigenic specificity (i.e. at the phenotypic level) determined by serological assays. Defined at the broad and split level (closely related but distinct specificities). Broad antigens are given in parenthesis after the split antigen. Many HLA alleles have the same serological specificity and conversely, many HLA alleles have no serological equivalent.	A1
Low	Molecular based HLA typing result corresponding to the first field of HLA nomenclature.	A*01
Intermediate	Molecular based HLA typing result that includes a group of alleles sharing the same first field but excludes some other alleles with the same first field. Sometimes include a minimum number of alleles with a different first field to the rest of the group.	A*01:01/01:0/ 01:14/36:04
High	Molecular based typing result that contains a set of alleles that encode the same protein sequence for the peptide binding region of the HLA molecule and excludes non-expressed HLA alleles. Includes groups of alleles with a P designation (encode same protein sequence in exon 2 and 3 for HLA class I and exon 2 for HLA class II) Includes groups of alleles with a G group designation (same nucleotide sequence across the exons encoding for peptide binding region). Does not include non-expressed alleles with same nucleotide sequence.	A*01:01P[a] A*01:01:01G[b]
Allelic	Molecular based tying result consistent with a single allele, defined as a unique nucleotide sequence for a gene and using all fields in the current allele name and defined in a given version of the WHO HLA Nomenclature Report.	A*01:01:01:01 A*02:07

[a] 01:01:01:01/01:01:02/01:01:03/01:01:04/01:01:05/01:01:06/01:01:07/01:01:08/01:01:09/ 01:01:10/01:01:11/01:01:12/01:01:13/01:32/01:37/01:45
[b] 01:01:01:01/01:01:01:02N/01:04N/01:22N/01:32/01:34N/01:37/01:45

NIMA:

The maternal HLA not inherited by the foetus. For example, if the CB donor *HLA-A* type is *A*01, 03* and the CB donor mother is *A*01, 02*, the CB has inherited *A*01* from the mother and but has not inherited the mother's *A*02*. The *A*02* is the NIMA. The NIMA is informative if the mother is heterozygous and the NIMA is mismatched to the CB IPA.

Phenotypes:

- **Inherited phenotype:** The expressed HLA profile of both HLA A~B~DRB1 haplotypes.

- **Virtual phenotype (VP):** CB inherited HLA phenotype with 1, 2 and/or 3 CB HLA at *HLA-A*, *-B* and/or *–DRB1* substituted for the corresponding NIMA.

Donor-recipient HLA matching:

- **Inherited HLA match:** The number of *HLA-A*, *-B* (traditionally at antigenic or intermediate resolution) and *–DRB1* (traditionally at high resolution) donor-recipient matches, determined as a count of six (6/6, 0 mismatch; 5/6, 1 mismatch; 4/6 2 mismatch; 3/6, 3 mismatch). A 6/6 is designated an inherited full match (IFM). Matching which additionally includes *HLA-C* is determined as a count of eight and can be based on low or high resolution.

- **NIMA match (NIMA+):** A donor-recipient HLA MM that is matched to the donor NIMA. For example, if the recipient is *A*01, 02*, the CB and recipient are

mismatched at *A*02*. If the CB NIMA is *A*02*, the HLA MM is designated a NIMA+.

- **Virtual full match (VFM):** When each of the donor-recipient MMs are matched to the NIMA. These include 5 inherited HLA matches plus 1 NIMA match (5/6 + 1 NIMA), four inherited HLA matches plus 2 NIMA matches (4/6 + 2 NIMA) and three inherited HLA matches plus 3 NIMA matches (3/6 + 3 NIMA).

2.4 Patients, donors and mothers

2.4.1 Active donors in the NHS-CBB

At the time of this research, 21,020 CBUs were banked with the NHS-Cord Blood Bank (CBB) and listed with the British Bone Marrow Registry (BBMR). Maternal HLA types were available for a proportion of these CB donors (*n*= 4,671).

NHS-CBB methods for CB collection, processing, testing, freezing and storage have been described previously elsewhere (Armitage 1999, Davey 2004, Navarrete 2009). Mothers were interviewed to obtain informed written consent for clinical and/or research use and the testing of CB in two stages. Consent to collect, evaluate and process CB and place it in short term storage was obtained prior to active labour. Consent for testing, use and long-term storage was obtained post-labour.

2.4.2 Donor-recipient transplant cohort

The NHS-CBB issued 499 CBUs between 1999 and June 2014, both nationally and internationally, to patients requiring CBT using one (single CBT) or two (double

CBT) units. Recipient disease indications comprised malignant and non-malignant types.

Demographic and clinical outcome data was available from multiple sources. As part of this research, a collated database containing all relevant information was developed. Recipient demographics and clinical outcome were available from Eurocord and transplant centres have previously provided written consent for the NHS-CBB to receive this data. Recipients (or their guardians) provided written consent for research. Additional recipient demographics were available from hard copies of patient files (stored at NHSBT) or from the laboratory information management system, Hematos (Savant). Demographics of mothers and their CBUs were available from Hematos or from hard copies of medical questionnaires.

2.4.3 Healthy male platelet donors

Whole blood collected in EDTA was obtained from NHSBT male platelet donors for DNA extraction to be used as controls in quantitative-polymerase chain reaction (q-PCR) assays. Donors were bled for the purpose of HLA typing by the clinical histocompatibility and Immunogenetics (H&I) laboratory and aliquots were taken of surplus blood not required. HLA typing results and gender of these donors were available from Hematos. Consent for research is obtained from blood donors.

2.5 Sample preparation

2.5.1 Whole blood DNA extraction

DNA was extracted from whole blood in EDTA using the QiaAmp DNA Maxi kit, as per the manufacturer's spin protocol. Briefly, 5 millilitres (ml) of whole blood was added to a 50ml conical tube (Fisher Scientific) containing 500µl QIAGEN Protease and mixed. 6ml of buffer AL was added, mixed by inverting 10 times and vigorous shaking for 2 minutes. The mixture was heated using a water bath to 70°C for 10 minutes to allow DNA lysis to occur. 5 ml of molecular grade ethanol was added and mixed as before. The mixture was spun in a QiaAmp Maxi column at 1850 x g for 3 minutes. The filtrate containing impurities was discarded, and further impurities were removed with two wash steps using buffer AW1 and AW2 and spun at 4500 x g for 1 minute and then 15 minutes. The Maxi column was transferred into a new 50 ml conical tube and the DNA was eluted using 600 microliter (µl) Buffer AE, with a 5-minute incubation step and centrifugation at 4500 x g for 2 minutes. To obtain highly concentrated DNA, the eluted DNA was reloaded onto the Maxi column and the elution step was repeated again.

2.5.2 Umbilical cord tissue DNA extraction

DNA extraction was performed using the QiAmp DNA Mini Kit (Qiagen) as per the manufacturer's spin protocol. Tissue from the inner layer of the cord tissue including the Wharton's jelly and blood vessels was dissected and homogenised in the manufacturer's lysis buffer. Due to the general age and physiology of the cord tissue samples, it was particularly difficult to extract high quality genomic DNA and results were inconsistent (dependent on starting material). Twenty-four-hour lysis with proteinase K resulted in a higher yield of genomic DNA when compared to 3-hour

lysis (12.3 nanogram (ng)/µl and 4.9 ng/µl respectively). Starting tissue weight had no effect on the concentration of genomic DNA extracted (data not shown). Personal observation suggested the highest yields of genomic DNA were achieved when thawed tissue appeared pink in colour (indicative of freezing soon after collection). Poor 260 nm/280 nm wavelength absorption wavelengths were also observed and it was necessary to purify genomic DNA prior to downstream molecular analysis.

2.5.3 DNA purification

Poor quality DNA samples were purified using Agencourt AMPure XP (Beckman Coulter) magnetic beads, as per the manufacturer's instructions. Briefly, beads were equilibrated to room temperature and added to DNA in a 1µl : 1.8µl ratio on a 96 well plate, mixed gently by pipetting up and down 10 times and incubated at room temperature for 5 minutes to allow the beads to bind to the DNA. The plate was placed onto a 96 well magnetic plate, left for 2 minutes to allow the bead-DNA complexes to move to the edge of the well and the clear liquid containing impurities was aspirated off. The bead-DNA complexes were then washed using 200µl of 80% molecular grade ethanol twice, with the ring of beads then allowed to air dry for 3 minutes. The plate was removed from the magnet and nuclease free water was added, mixed by pipetting up and down 10 times and the plate was incubated for 5 minutes to allow the beads and DNA to separate. The volume of water added was variable depending on the starting DNA concentration but ranged from 30 – 70µl. The plate was placed back onto the magnet for 2 minutes and the purified DNA was transferred to an Eppendorf tube.

2.5.4 DNA quantification

Sample absorbance of ultraviolet light was measured by spectrophotometry using a Nanodrop to determine sample concentration and quality. DNA concentration was calculated from the optical density at 260 nm. The ratio of absorbances at 260 nm (nucleic acids) and 280 nm (proteins) was used to assess purity and an A260/280 ratio of 1.8 was deemed optimal. The ratio of absorbance at 260 nm and 230 nm was used to check for other contaminants (for example from the nucleic acid extraction method) and an A260/230 ratio of 2.0 – 2.2 was deemed optimal.

Genomic DNA was diluted to the required concentration using distilled H_2O.

2.6 HLA reports and typing techniques

2.6.1 Recipient, CB and maternal HLA types

Recipient and CB HLA typing was performed at a minimum of low resolution at *HLA-A* and *-B* and at high resolution at *-DRB1* loci. Maternal HLA typing was performed to a minimum of low resolution at *HLA-A* and *-B* and *-DRB1* loci. All donor-recipient HLA types of transplanted pairs were confirmed. Initial CB and maternal HLA typing was completed internally by the H&I laboratory at Colindale by polymerase chain reaction (PCR)-sequence specific primers (PCR-SSP), PCR-sequence specific oligonucleotides (PCR-SSO) or PCR-sequenced based typing (PCR-SBT). Recipient HLA typing was performed externally in the majority of cases and typing data was provided by Eurocord. Hard copies of recipient files were checked for additional HLA typing information. If HLA typing of the

recipient was performed internally by NHSBT, the data was also downloaded from Hematos. CB and maternal HLA types were downloaded from Hematos.

The majority of recipients, CB donors and mothers did not have allelic resolution *HLA-A*, *-B*, *-C* and *-DRB1* types. For the purpose of this thesis, if recipient DNA was not available at Colindale, pre-transplant recipient DNA and/or allelic level HLA typing results were requested from UK transplant centres or the local H&I laboratory serving the transplant centre. For non-UK transplant centres, recipient allelic level HLA typing results were requested from Eurocord. Individual non-UK transplant centres were not contacted because the NHS-CBB is under agreement that all requests for data should be done via Eurocord, who are responsible for contacting the transplant centre. Retrospective allelic resolution *HLA-A*, *-B*, *-C* and *-DRB1* typing for recipients, CB donor and mothers was performed by myself as required and when samples were available (see below).

2.6.2   Sequenced based typing

Allelic resolution typing of *HLA-A*, *-B*, *-C* and *-DRB1* genes was performed using a commercial method, SBT Excellerator. All reactions were performed as per the manufacturer's instructions, unless otherwise stated. Thermal cycling was performed using a thermocycler and sequencing was performed by capillary electrophoresis using a genetic analyser.

An initial PCR amplification targeting the gene of interest was performed using a Long Range PCR Kit and SBT Excellerator amplification primers. Reactions mixes for 96 samples +10% additional dead volume and without template DNA were made as

one master mix for each HLA locus and these were aliquoted as a single reaction per well in a semi-skirted 96 well plate for each HLA locus. Amplification reactions containing approximately 50 ng of genomic DNA were prepared according to **Table 2.2** and cycling was performed according to **Table 2.3.** A non-template control (NTC) was included for all reactions. Amplification was confirmed by gel electrophoresis. Class I amplicons were 3.1 – 3.4 kb and class II amplicons were 3.7 – 4.8 kb in size.

Table 2.2 Class I and Class II amplification reaction mix per reaction.
Amplification was performed single-plex for each HLA gene. *HLA-A*, *–B* and *–DRB1* reaction mixes were made at half the volume given below. *HLA-C* reaction mixes were made at the recommended volume below. *Less than 50 ng of template DNA was added if the stock concentration was less than 25 ng/μl.

Component	Volume (μl)	Final concentration
Long Range PCR buffer with Mg2+	2.5	1X, 2.5 mM Mg^{2+}
dNTP mix (10mM each)	1.25	500 μM each dNTP
HLA amplification primers	1	-
Long Range PCR enzyme mix	0.4	2 units
Nuclease free H_2O	17.85	-
Template DNA	2	50 ng*

Table 2.3 Class I and class II thermal cycling amplification protocol. Time is shown in minutes and seconds.

Parameter	Temperature (^{0}C)	Class I time	Class II time
Initial denaturation	95	3:00	3:00
3 step cycling (x35):			
– Denaturation	95	0:15	0:15
– Annealing	65	0:30	0:30
– Elongation	68	3:00	5:00
Final extension	68	10:00	10:00

Hold	4	∞

Illustra ExoProStar was used to remove unincorporated primers and nucleotides post-amplification. Three µl ExoProStar was added to PCR products on ice, vortexed and incubated at 37°C for 15 min to activate the exonuclease I and alkaline Phosphatase, followed by 15 min at 80°C to inactivate the enzymes.

Sequencing was performed in both directions for exons 2 – 4 for *HLA-A*, *-B* and *-DRB1* and exons 1 – 7 for *HLA-C* as a minimum. After the first round of sequencing, if genotype ambiguities were present, additional reactions were performed using group specific primers (GSSPs) to resolve the cis/trans ambiguity. Additional exons (e.g. exon 1 and 5 for *HLA-B*) were sequenced if allele ambiguities were present. Sequencing reactions containing 1 µl product were prepared for $n + 10\%$ as shown in **Table 2.4** and cycle sequencing was performed as detailed in **Table 2.5.**

Table 2.4 Class I and Class II sequencing reaction mix. Individual sequencing reactions were performed single-plex for each exon in each direction.

Component	Volume (µl)
BDT Ready reaction mix, 2.5X	1
BDT Buffer, 5X	1.5
Nuclease free H_2O	5.5
Sequencing primer	1
Purified PCR product	1

Table 2.5 Class I and class II thermal cycling protocol for cycle sequencing.
Time is shown in minutes and seconds.

Parameter	Temperature (^{0}C)	Time
Initial denaturation	96	0:10
3 step cycling (x25):		
– Denaturation	96	0:10
– Annealing	50	0:10
– Elongation	60	2:00
Hold	4	∞

Dye terminators, dNTPs, salts and other low molecular weight materials from sequencing reactions removed from products using a gel filtration system. Sequencing products were loaded onto a 96 well Performa DTR plate, spun for 5 minutes at 850 x g and the elute containing purified sequencing products was transferred to an optical 96 well plate. Purified sequencing products were run on a sixteen capillary 3130 genetic analyser.

HLA alleles were assigned using SBTengine software (Gendx), which compares the sample sequence with the most recent reference sequence from the IMGT HLA database (http://www.ebi.ac.uk/imgt/hla).

2.6.3 *In silico* HLA allele prediction

The feasibility of predicting HLA alleles from low resolution HLA types using a computational method was investigated by comparing molecularly defined alleles to imputed alleles from first field HLA. The HLAMatchmaker four-digit

convertor (http://www.hlamatchmaker.net), designed by Duquesnoy (University of Pittsburgh), converts serologically defined HLA antigens to the most likely allele based on allele frequencies, available from the IMGT/HLA database, from four major population groups: European Caucasoid, African-American, Hispanic and Asian.

2.7 Quantification of microchimaerism by real-time quantitative-PCR

CB DNA was tested for the presence of MMc by q-PCR targeting HLA polymorphisms and using SYBR® Green chemistry (Life Technologies). Assays targeted HLA polymorphisms informative for NIMA and quantification was from the quantification of the starting template using an endogenous control, human cell kinase (HCK) and an absolute quantification method.

2.7.1 Primers

HLA primer sequences were kindly provided by Dr Michael Eikmans, Department of Immunohematology and Blood Transfusion, Leiden University Medical Centre, the Netherlands (Drabbels*, et al* 2011, Eikmans*, et al* 2014). Assay targets were: *HLA-A*01, -A*02,* -A9*, -A*11, -A*24, -A*30, -A*33, -B*08, -B*27, -B*35* and *-DRB1*03.* High performance liquid chromatography (HPLC) purified primers were synthesised by Eurofins Genomics. Details of primer sequences, targets and specificity are given in chapter 6. Primers targeting the endogenous control, HCK, were obtained directly from Sigma-Aldrich (Gene ID: 3055, RefSeqID: NM_001172129). All primers were ordered lyophilised and were reconstituted in nuclease free water to a stock concentration of 100µM (100 picomoles per µL).

2.7.2 HLA and HCK amplification

The q-PCR was performed using 200 ng of purified template DNA (see **section 2.5.3**), added to SYBR® Select Master Mix and both forward and reverse primers **(Table 2.6)**. Reactions were performed in an optical 96-well reaction plate and with an optical seal. The thermal cycling protocol consisted of target

amplification followed by a continuous dissociation curve **(Table 2.7)**. All changes in temperature were achieved with a ramp speed of 1.6^0C per second to increase sensitivity (fast ramp speeds have been associated with lower sensitivity and higher variability (Hilscher, *et al* 2005)). Reactions were performed on a QuantStudioTM 7 Flex Real-Time PCR System (Applied Biosystems).

Table 2.6 Master Mix for HLA q-PCR. *Less than 200 ng of template DNA was added if the stock concentration was less than 100 ng/µl.

Component	Volume (µl)	Concentration
SYBR® Select Master Mix	10	2X
Forward and reverse primer mix	0.3	Variable
DNase free water	5.7 - 7.7	-
Template DNA	2 – 4	200ng*

Table 2.7 HLA q-PCR cycling protocol

Parameter	Temperature (^{0}C)	Time
Initial denaturation	95	08:30
Cycling (x40)		
– Denaturation	95	0:15
– Annealing	60	1:00
– Extension	55	1:00
Dissociation curve	55 – 95	Continuous

2.7.3 Primer specificity

HLA primer specificity was tested by setting up q-PCR reactions using DNA samples of male platelet donors positive and negative for the target of interest. A

panel of HLA typed male platelet donors was carefully constructed to represent a broad range of HLA types and overcome LD as much as possible **(Table 2.8)**. Specificity was determined by the presence of a single, narrow peak in the post-amplification dissociation curve and the absence of amplification in the non-template control (NTC) and samples negative for the target of interest. Cross reactivity was defined as the presence of a melting temperature that fell within the range of the target of interest.

Table 2.8 HLA types of the male platelet donor panel used to test HLA primer specificity. Donors were selected to represent a broad range of HLA types. HLA typing was previously performed by the H&I service laboratory using PCR-SSOP for clinical purposes.

Donor	*HLA-A*		*HLA-B*		*HLA-C*		*HLA-DRB1*	
1	*02	*66	*08	*41	*07	*17	*08	*13
2	*26	#	*14	*44	*07	*08	-	-
3	*02	*68	*07	*27	-	-	-	-
4	*03	*68	*07	*53	*04	*07	-	-
5	*02	*31	*40	#	*03	#	*01	*03
6	*03	#	*07	#	*07	#	-	-
7	*01	*02	*07	*44	*07	#	-	-
8	*02	*25	*13	*18	*06	*12	*01	*08
9	*01	#	*08	#	*07	#	-	-
10	*02	#	*49	#	*07	#	-	-
11	*02	*31	*44	*52	*02	*12	-	-
12	*30	#	*42	*49	*07	*17	*03	*07
13	*02	*74	*08	*45	*07	*16	-	-
14	*01	*03	*56	*57	*01	*06	-	-
15	*01	#	*41	#	*17	#	-	-
16	*31	*32	*15	*50	*03	*06	-	-
17	*02	*31	*57	*58	*06	*07	-	-
18	*32	#	*27	#	*01	#	-	-
19	*29	#	*44	#	*16	#	*07	#
20	*11	#	*44	*51	*15	*16	-	-
21	*02	#	*15	#	*03	#	*07	*13
22	*11	*24	*55	#	*03	#	-	-
23	*03	*11	*35	#	*04	#	-	-
24	*01	*02	*08	*47	*06	*07	-	-
25	*02	*03	*37	*38	*06	*07	-	-
26	*02	*24	*40	*48	*03	*08	-	-
27	*01	*32	*08	*14	*07	*08	-	-
28	*02	*24	*39	*51	*07	*14	-	-
29	*24	*25	*44	#	*05	#	*01	*07
30	*02	*31	*35	*78	*04	#	-	-
31	*24	#	*55	*57	*03	*06	-	-
32	*11	*32	*15	#	*03	#	*04	#

2.7.4 Artificial spiking experiments and standard curves

Serial dilutions were generated by artificial spiking experiments and were used to generate a standard curve. Standard curves were used to evaluate the assay performance to in calculations of quantification. A male platelet donor positive for the HLA target of interest (the minor component) was serially diluted five times by 1:10 with an additional 0.05% dilution, in a background DNA that was negative for the target of interest (the major component) and from a starting concentration of 100 ng/µl **(Figure 2.1)**. 200 ng of total template was added to each q-PCR reaction.

Figure 2.1 Serial dilution of a sample positive for the target into a background of DNA negative for the target of interest.
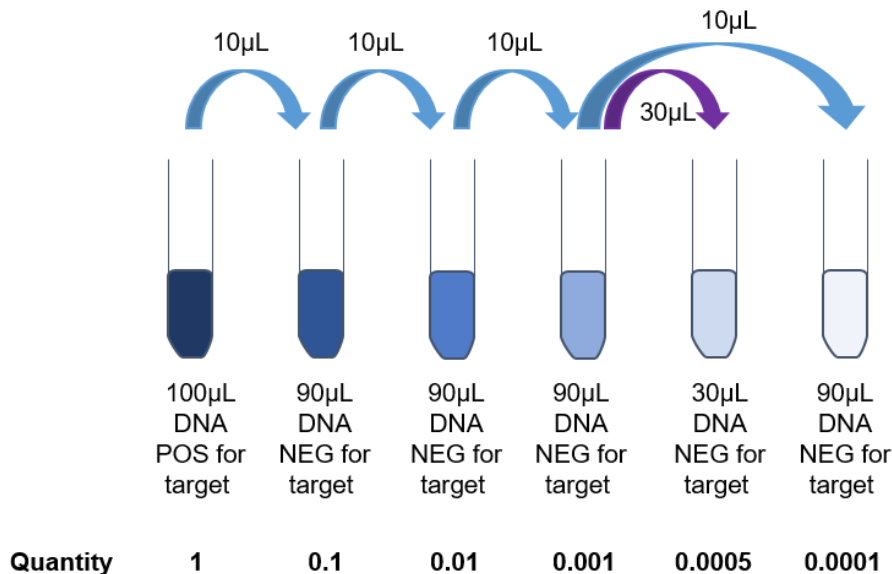
Amplification efficiency can be calculated from the standard curves of q-PCR reactions with a serial dilution of template quantity. The quantitation cycle (C_q) is defined as the cycle at which the fluorescence crosses the background level and is directly proportional to the amount of starting template. In this way, the amount of starting target DNA can be quantified and two samples that differ by 1:2 in template quantity should differ by one C_q value. The starting quantities of a series of dilutions can therefore be plotted against their respective C_q values to generate a standard curve. A standard curve whereby each cycle corresponds to a doubling of PCR product has a slope of –3.32, although in principle this does not occur. The actual amplification efficiency (E) of a q-PCR reaction is optimised to have an efficiency of 90 – 110% and is calculated from the slope of the standard curve by using the following formula:

$$E = 10^{-1/\text{slope}}$$

2.7.5   MMc quantification assay design

MMc in CB DNA was quantified by an absolute quantification method that determined the amount of NIMA HLA template relative to the amount of HCK template present in the sample. The amount of HLA and HCK template were quantified from standard curves that were run on each plate **(Figure 2.2)**. Individual q-PCR plates were set up for each NIMA HLA target. Each CB sample containing an unknown quantity of NIMA template was tested in triplicate for NIMA HLA amplification and in duplicate for HCK amplification and eight CB samples were tested per plate. Controls for HLA and HCK amplification and quantification were a reference sample positive for the target, a sample negative for the target and a NTC.

Figure 2.2 qPCR 96 well plate layout for MMc testing. Key: Blue, HLA; Orange, HCK; *(S),* Standard serial dilutions of male platelet donor DNA positive for target of interest or HCK, *(U),* Unknown CB DNA; POS, positive DNA for target of interest; NEG, negative DNA for target of interest; NTC, non-template control*.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	*(S)* Target HLA standard curve						*(U)* CB #1 HLA			*(U)* CB #1 HCK		NEG HCK
B							*(U)* CB #2 HLA			*(U)* CB #2 HCK		
C							*(U)* CB #3 HLA			*(U)* CB #3 HCK		NTC HLA
D	*(U)* HCK of HLA standard curve samples						*(U)* CB #4 HLA			*(U)* CB #4 HCK		
E							*(U)* CB #5 HLA			*(U)* CB #5 HCK		
F	*(S)* HCK standard curve						*(U)* CB #6 HLA			*(U)* CB #6 HCK		NTC HCK
G							*(U)* CB #7 HLA			*(U)* CB #7 HCK		
H	POS HLA		POS HCK		NEG HLA		*(U)* CB #8 HLA			*(U)* CB #8 HCK		

2.7.6 Analysis

Analyses of q-PCR were performed using the Quant Studio 7 version 1.3 software and in the corresponding Applied Biosystems™ qPCR analysis app available in the Thermofisher cloud (https://www.thermofisher.com).

2.8 Computational and statistical analyses

2.8.1 Generation and characterisation of virtual phenotypes

2.8.1.1 HLA allele and haplotype segregation

HLA-A, *-B* and *–DRB1* HLA types of mothers and their CBUs were compared to define alleles using a pedigree analysis algorithm in collaboration with a bioinformatician, John Ord, who then wrote the SQL command in MS Access. HLA typing was previously performed by the H&I laboratory at NHSBT Colindale. The intermediate and high resolution HLA typing results were collapsed back to the

first field to remove ambiguous typing data and to permit statistical analyses. HLA alleles in common between the CBU and mother (identical by descent) were identified as the IMA. The CBU HLA alleles not shared with the mother were identified as the IPA. The maternal HLA alleles not shared with the CBU were identified as the NIMA. An example is shown in **Figure 2.3, Example 1A**. At each HLA locus, NIMA could only be identified and therefore deemed informative if the mother was heterozygous and the NIMA was mismatched to the CBU IPA at first field resolution. An example of a CBU and maternal pair where the NIMA was non-informative is shown in **Figure 2.3, Example 2A**. The algorithm was validated by comparison with manual assignment of NIMA using Microsoft Excel, which was performed by myself.

Figure 2.3 Examples of CBU and maternal pairs (1) informative and (2) non-informative for NIMA at *HLA-A*, *-B* and *–DRB1* and the generation of CBU virtual phenotypes. (A) Comparison of CBU and maternal HLA types allowed the identification of allele segregation and therefore the NIMA (yellow), unless the mother was homozygous or a 0 MM to the CBU. **(B)** Inherited CBU HLA were substituted for the corresponding NIMA at 1 or more HLA loci. Maximum number of virtual phenotypes per CBU is 26: 1 NIMA substitution, 6; 2 NIMA substitutions, 12 and 3 NIMA substitutions, 8 virtual phenotypes. This is reduced if the NIMA is non-informative.

A. Identify NIMA

B. Generate virtual phenotypes

Example 1. *HLA-A*, *-B* and *–DRB1* informative for NIMA

	HLA-A	HLA-B	HLA-DRB1
CBU	01,24	08,40	03,09
Mother	01,02	08,15	03,13
IPA	24	40	09
IMA	01	08	03
NIMA	02	15	13

1 NIMA substitution	2 NIMA substitutions	3 NIMA substitutions
A – B – DRB1	A – B – DRB1	A – B – DRB1
02,24 - 08,40 - 03,09	**02**,24 - **15**,40 - 03,09	**02**,24 - **15**,40 - 09,**13**
01,**02** - 08,40 - 03,09	01,**02** - **15**,40 - 03,09	**02**,24 - **15**,40 - 03,**13**
01,24 - **15**,40 - 03,09	**02**,24 - 08,**15** - 03,09	01,**02** - **15**,40 - **13**,09
01,24 - 08,**15** - 03,09	01,**02** - 08,**15** - 03,09	01,**02** - **15**,40 - 03,**13**
01,24 - 08,40 - 09,**13**	**02**,24 - 08,40 - **13**,09	**02**,24 - 08,**15** - 09,**13**
01,24 - 08,40 - 03,**13**	01,**02** - 08,40 - **13**,09	**02**,24 - 08,**15** - 03,**13**
	02,24 - 08,40 - 03,**13**	01,**02** - 08,**15** - **13**,09
	01,**02** - 08,40 - 03,**13**	01,**02** - 08,**15** - 03,**13**
	01,24 - **15**,40 - 09,**13**	
	01,24 - 08,**15** - **13**,09	
	01,24 - **15**,40 - 03,**13**	
	01,24 - 08,**15** - 03,**13**	

Example 2. *HLA-A* and *-B* informative, *–DRB1* non-informative for NIMA

	HLA-A	HLA-B	HLA-DRB1
CBU	01,32	08,49	01,03
Mother	01,29	08,44	01,03
IPA	32	49	-
IMA	01	08	-
NIMA	29	44	-

1 NIMA substitution	2 NIMA substitutions
A – B – DRB1	A – B – DRB1
29,32 - 08,49 - 01,03	**29**,32 - **44**,49 - 03,01
01,**29** - 08,49 - 01,03	01,**29** - **44**,49 - 03,01
01,32 - **44**,49 - 01,03	**29**,32 - 08,**44** - 03,01
01,32 - 08,**44** - 01,03	01,**29** - 08,**44** - 03,01

2.8.1.2 Generation of virtual phenotypes

A VP algorithm was developed in collaboration with John Ord. I designed the strategy of generating the virtual VPs and he implemented this using SQL in MS Access. VPs were generated by substituting 1, 2 or 3 CBU HLA for the corresponding NIMA at *HLA-A*, *-B* and/or *–DRB1* **(Figure 2.3, Example 1B)**. If the CBU was informative for NIMA at a given HLA locus (allele *x*, allele *y*), a substitution for NIMA was made at allele *x*, to yield one VP and at allele *y*, to yield a second VP. If *HLA-A*, *-B* and *–DRB1* were all informative for NIMA, 26 VPs per CBU were generated. The number of VPs was reduced if the NIMA was non-informative **(Figure 2.3, Example 2B and Table 2.9)**. The algorithm was validated by myself by the comparison with manual assignment of VPs in Microsoft Excel and against a previously published example (Van der Zanden, *et al* 2014).

Table 2.9 Maximum number of virtual phenotypes per CBU according to the number of informative NIMA and substitutions made at *HLA-A*, *-B* and *–DRB1*. Sub., substitution.

	Number of virtual phenotypes			
Informative NIMA	**1 sub.**	**2 sub.**	**3 sub.**	**Total**
0 NIMA	0	0	0	0
1 NIMA	2	0	0	2
2 NIMA	4	4	0	8
3 NIMA	6	12	8	26

2.8.2 Searches for HLA and NIMA matched CB donors

Searches for an IFM (6/6, 0 mismatch), 5/6 (1 mismatch) and 4/6 (2 MMs) and VFM (1 – 3 NIMA) were performed on the UK-BBMR and Bone Marrow Donors Worldwide (BMDW) registries. At the time of search, 4,707 of the total 21,020 UK-BBMR and 26,735 of the total 621,893 BMDW listed CB donors had maternal HLA typing available. HLA matching was based on *HLA-A* and *–B* at antigenic or intermediate resolution and *–DRB1* at high resolution level. Only CB donors with the required level of HLA typing were recorded as potential donors. The BMDW CBU Match Program was used to identify inherited HLA matches using the "identical and one allele/antigen mismatch" and "two antigen/allele MMs" functions.

VFMs were identified using the BMDW NIMA Match Program with "Identical Matches" and "Include 2 and 3 NIMA in match" functions selected. If multiple NIMA matched donors were identified for a patient, the donor with the highest level of inherited HLA matching was selected.

Searches for single CBUs, with adequate total nucleated cell (TNC) dose, were performed on a subset of patients weighing 50 kg or less (*n*= 204). The median weight of these patients was 16 kg. IFMs were identified first. Patients with only a mismatched donor were evaluated for a NIMA match. Patients without a NIMA match were assessed for a 5/6 or 4/6 inherited HLA match. Only donors which met a minimum TNC dose of 2.5×10^7 per kg for malignant diseases and 4.0×10^7 per kg for non-malignant diseases (Rocha and Gluckman 2009) were recorded as potential donors.

2.8.3   Retrospective analyses of clinical outcome after NIMA matched CBT

The clinical outcomes of CBT were compared between HLA matched, NIMA matched and non NIMA matched categories in a retrospective analysis.

Analyses were performed in collaboration with an NHSBT statistician, Elinor Curnow. I coded the dataset demographics and determined the HLA matching status between donors and recipients. I also coded the time-to-event outcomes data and we discussed and resolved discrepancies in data reporting, non-sense values and unusual cases together. I performed preliminary outcomes analyses between the groups and identified variables significantly associated with outcomes. Elinor confirmed and extended these analyses and performed the sample size analysis.

2.8.3.1   Definitions of statistical endpoints

The primary endpoint was 5 year OS. Secondary endpoints were TRM, relapse, myeloid engraftment, aGvHD and cGvHD. Definitions of endpoints and descriptions of the censoring, competing events and the patients evaluated are shown in **Table 2.10**.

The experience of the various outcomes, dates of onset and other relevant information e.g. grade of aGvHD were provided by Eurocord. All data were checked for discrepancies and non-sense values. Time-to-event (or time-to-censorship) was calculated by the time difference between the date of transplant and the date of the event (or date of last follow-up) using the DATEDIFF function in Microsoft Excel 2013.

Table 2.10 Definition of transplant endpoints

Endpoint	Definition	Censoring	Competing event	Patients
5-year OS	Probability of survival at 5 years post-transplant irrespective of disease.	Patients alive at date of last follow up.	None	All patients
5-year TRM	Death from any cause apart from the original disease.	Patients alive without relapse at date of last follow up.	Relapse	All patients
5-year Relapse	Recurrence of original disease.	Patients alive and without relapse at date of last follow up.	Death without evidence of relapse.	Patients with malignant disease
Myeloid engraftment	Absolute neutrophil count > 0.5×10^9/l on three consecutive days.	Patients alive and without myeloid engraftment at date of last follow up.	Death before myeloid engraftment.	All patients
aGvHD	Clinical diagnosis that may be supported by biopsies. Graded I-IV based on organ involvement and usually occurs within 100 days.	Patients alive and without aGvHD at date of last follow up.	Primary or secondary graft failure. Death without aGvHD.	All patients
CGvHD	Clinical diagnosis supported by biopsies and exclusion of other diagnoses. Usually occurs after 100 days.	Patients alive and without cGvHD at date of last follow up.	Primary or secondary graft failure. Death without aGvHD.	Patients alive at day 100

2.8.3.2 Statistical analyses

OS at 5-years post-transplant was determined using the Kaplan-Meier estimate and the log-rank test was used to compare estimates between match categories. The incidence of TRM at 5 years, relapse at 5 years, myeloid engraftment, aGvHD at 100 days and cGvHD at 5 years post-transplant were calculated using the cumulative incidence estimator and these were compared between match categories using Gray's test of regression (Scrucca, *et al* 2010). Death due to non-transplant related causes and relapse were competing risks for TRM; death from any cause was the competing risk for relapse; death from any cause and graft

failure prior to the outcome of interest were competing risks for engraftment, aGvHD and cGVHD. Cumulative incidence of cGvHD was estimated conditional on transplant survival to 100 days.

The relationship between match category and each outcome was further investigated using Cox regression (OS) and the Fine-Gray regression model (all other outcomes) (Scrucca, *et al* 2010). Hazard ratios were reported for Cox regression and sub-distribution hazard ratios were reported for Fine-Gray regression models. Results were adjusted for risk factors associated with CBT outcomes: log(TNC dose), recipient age, conditioning regimen (MAC vs. RIC), year of transplant and whether the recipient was being treated for a malignant disease. Regression analyses were repeated for the sub-group of cases with at least one HLA mismatch, adjusting for the number of MMs (1-2 vs. 3-4) and whether there was a MM at *HLA-C*, as well as the other risk factors previously listed.

The median time to acute or chronic GvHD was used in cases where acute or chronic GvHD was reported but the date of GvHD occurrence was unknown. Recipients for whom risk factors were not reported were excluded from regression modelling.
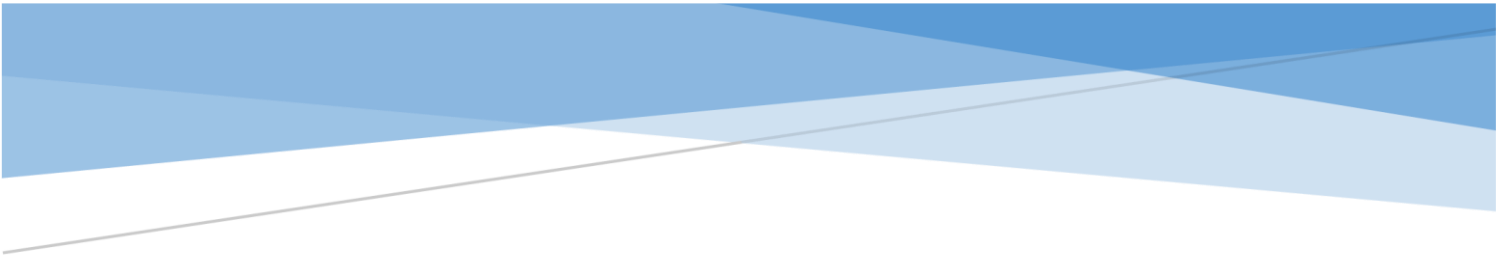
Statistical analyses were undertaken using SAS/STAT version 9.4 (SAS Institute Inc.) and R version 3.2.0 (R Foundation for Statistical Computing) and SPSS version 23 (IBM).

2.8.3.3 Sample size required to reach a statistically significant difference in the primary end-point

The sample size required to determine a statistically significant difference in OS between NIMA matched and non-NIMA matched cases was calculated using a method based on the log-rank test (Collett 2015). An 80% power and 5% significance was assumed and a ratio of NIMA-matched to non-NIMA matched cases of 1:7.

2.8.4 Statistical differences between categorical variables

Differences between categorical values were tested by the X^2 or Fisher's Exact test statistic in SPSS. The Z test with corrected P values using the Bonferroni method was used to compare column proportions. P values are two sided.

Chapter 3 Maternal HLA typing of banked CBUs to identify NIMA HLA and generate virtual phenotypes available for donor searches

Key points:

- Development of in-house algorithms to identify the IMA/IPA and NIMA alleles and haplotypes of CBUs and to generate VPs.
- 66,225 VPs (14-fold increase) were generated from 4,671 CBUs with maternal HLA types and of these 52,875 were unique.
- 48,221 of these VPs were not represented as inherited phenotypes in the total 21,020 banked CBUs and thus increased the total number of unique phenotypes in the NHS-CBB to 65,046 (3.9-fold increase).
- HLA-A/-B/-DRB1 allele and haplotype frequencies of the NHS-CBB mothers and CBUs were diverse.

3.1 Introduction

A greater degree of HLA disparity is tolerated in CBT but HLA MMs are still associated with an increased risk of aGvHD and mortality (Eapen, *et al* 2011b, Flomenberg, *et al* 2004, Furst, *et al* 2013, Jagasia, *et al* 2012, Kanda 2013, Lee, *et al* 2007, Woolfrey, *et al* 2011). Despite the listing of over 700,000 CB donors with BMDW, a 6/6 HLA matched CB donor is available for only 38% of Caucasian, 16 - 20% of Asian and 5 - 6% of Black patients (Barker, *et al* 2010b, Gragert, *et al* 2014). An improvement in the availability of HLA matched CBUs is therefore clinically needed. One method to increase the number of phenotypes available for CB donor searches is to increase the number of HLA typed banked CBUs (Querol, et al 2009). This resource intensive approach may not be economically feasible, especially given that many banks are struggling to maintain financial stability (Magalon, et al 2015).

A possible alternative or complementary approach is to identify permissible HLA MMs such as NIMA, which have shown improved clinical outcomes compared to non-NIMA HLA MMs in retrospective analyses (Rocha, *et al* 2012, van Rood, *et al* 2009). In CBT, NIMA matching has previously occurred by chance in approximately 7 - 10% of HLA mismatched transplants but HLA typing the mothers of banked CBUs would enable the prospective provision of a NIMA match, although the probability of this is currently unknown. It will likely depend on the frequency of the NIMA HLA in the donor population searched and whether it is found in LD with the HLA at other patient/CB HLA loci. Population specific HLA allele and haplotype frequencies have previously been used to model HLA match rates for patients requiring HSCT (Gragert, *et al* 2014) and similarly,

knowledge of NIMA HLA allele and haplotype frequencies could be a useful tool for modelling NIMA match rates. The NHS-CBB has been completing maternal HLA typing of banked CBUs but a population genetics analysis of CBUs and mothers has not yet been performed.

A NIMA match whereby each donor-recipient HLA MM at *HLA-A*, *-B* and/or *-DRB1* is a match to the donor NIMA is termed a VFM (see **M&M section 2.3**) (Van der Zanden*, et al* 2014). VFMs can be identified from VPs of the CBU, generated by the substitution of 1 - 3 inherited (IMA/IPA) CBU HLA for the corresponding NIMA at *HLA-A*, *-B* and/or *–DRB1*. This identifies all the possible positions where a NIMA match could occur and up to 26 VPs can be generated for each CBU with a maternal HLA type (Van der Zanden*, et al* 2014, van Rood*, et al* 2009). The NHS-CBB and others have recently started to list maternal HLA types with BMDW and in 2012 an algorithm that searches for VFMs for patients, the BMDW NIMA Match Program, was launched.

There is very little information regarding VPs and the probability of identifying a VFM in the literature since a minority of CBBs are performing maternal HLA typing. In the only published study, van der Zanden and colleagues analysed the maternal HLA of 6,827 CBUs in the NYCB (Van der Zanden*, et al* 2014). They reported a yield of 122,180 VPs, which was lower than 177,502 expected if 26 VPs were generated for each of the 6,827 CBUs. A VP is not generated if the NIMA is non-informative at an HLA locus, either because the mother is homozygous or a 0 HLA MM with the CBU (Van der Zanden*, et al* 2014). The authors did not explore non-informative NIMA in any detail and left many questions unanswered. Since it is unlikely that resources will be available to perform maternal HLA

typing for all of the currently banked CBUs, selection should be based on those most likely to be informative for NIMA and thus able to yield the maximum potential of 26 VPs. Furthermore, is not known whether there are differences in the number of VPs between different populations, or whether these VPs provide an increase to the pool of unique phenotypes available for donor searches.

3.2 Aims

The aim of this chapter was to define the *HLA-A*, *-B* and *-DRB1* NIMA of banked CBUs with maternal HLA types available, to generate VPs derived from the NIMA and to evaluate whether these increase the number and diversity of HLA phenotypes available for donor searches. This required the development of two *in house* algorithms to identify the CBU IMA/IPA/NIMA HLA alleles and haplotypes and to generate the VPs, which was carried out in collaboration with a bioinformatician, John Ord.

Several questions were formulated and answered to address the aim of this chapter **(Figure 3.1)**. The first question, "what is the HLA diversity of mothers and their CBUs in the NHS-CBB?" was evaluated by an analysis of HLA alleles and A~B~DRB1 haplotypes and their frequencies amongst the different populations. HLA types of mothers and CBUs were compared to identify allele segregation and thus the IMA/IPA and NIMA HLA haplotypes. To the best of the author's knowledge, this is the first time donor registry HLA haplotype frequencies in multiple populations have been reported without the need to estimate HLA haplotypes using models such as Expectation-Maximisation (E-M) and instead

were determined through allele segregation, as reported for small scale family studies (Gragert, *et al* 2013, Tang, *et al* 2007, Tu, *et al* 2013).

The identification of CBU IMA/IPA and NIMA HLA haplotypes led to the ability to answer the second question, "when and how often are the CBU NIMA non-informative?" The occurrences of maternal homozygosity and CBU-maternal 0 MM at *HLA-A*, *-B* and *-DRB1* were determined and results were compared between the broad populations. Identification of informative NIMA permitted the substitution of 1 - 3 CBU HLA for the corresponding informative NIMA at *HLA-A*, *-B* and/or *-DRB1* to generate VPs. The third question was "how many VPs are present in the NHS-CBB and do these increase the donor pool available for searches?" The total number of VPs generated from 4,671 CBUs was compared to the maximum expected, 26-fold increase, if all CBUs were informative for three NIMA at *HLA-A*, *-B* and *-DRB1*. Unique VPs were identified and the value of these to the donor pool of the NHS-CBB was evaluated by determining whether these VPs represented new phenotypes that were not yet present as inherited phenotypes.

Figure 3.1 Chapter 3 aim and the questions proposed to address this aim

What is the HLA diversity of mothers and CBUs?

- Proportion that are non-Caucasian
- Unique haplotypes
- New unique haplotypes provided by NIMA
- Haplotype frequencies
- Rare haplotypes

Aim:
To define the *HLA-A, -B* and *-DRB1* NIMA of banked CBUs with maternal HLA types available, to generate virtual phenotypes derived from the NIMA and to evaluate whether these increase the number and diversity of HLA phenotypes available for donor searches.

When and how often are the CBU NIMA non-informative?

How many virtual phenotypes are in the NHS-CBB and do these increase the donor pool available for searches?

- Most frequent reason for the NIMA being non-informative
- Proportion of CBUs non-informative at one or more HLA locus
- Comparison of non-informative NIMA between HLA loci
- Comparison between donor ethnicities

- Quantification of the total virtual phenotypes
- Number of unique virtual phenotypes
- Virtual phenotypes not yet represented as IPs in the NHS-CBB

3.3 HLA and ethnic diversity of mothers and their CBUs

3.3.1 Mapping self-reported ethnicities to broad population groups

There were 4,671 CBUs with maternal HLA types of the total 21,020 banked in the NHS-CBB. Self-reported ethnicities were mapped to broad population groups with the aim to 1) create groups which made best use of the sample size available to allow comparisons between the groups, 2) reflect the population diversity in the NHS-CBB and 3) group on a similar basis to population groups used worldwide to allow comparison with other registry datasets such as the American National Marrow Donor Program (NMDP). Broad populations chosen were Caucasian (CAU), Asian (ASI), South and East Asian (SEA), Black (BLK) and Mixed (MIX). Just under 40% of CBUs mapped to a non-Caucasian or mixed broad population (ASI, 16.9%; SEA, 1.0%; BLK, 5.5%; MIX, 13.0%). An additional 1.8% were grouped as other ethnicity (OE) and 1.3% were unknown or not declared (UNK) **(Table 3.1)**.

Table 3.1 Self-reported ethnicities and their mapping to broad population groups. Broad populations and their percentages of the total population included are shown in bold. Self-reported ethnicities and their percentages within the mapped broad group are shown below the broad populations. Total CBUs *n*= 4, 671.

Population	N	%
Caucasian (CAU)	**2,831**	**60.6**
Caucasian	1,761	62.2
UK/North Europe	653	23.1
White British	7	0.2
South Europe White	35	1.2
Mediterranean White	15	0.5
Middle East Arab	40	1.4
Jewish	43	1.5
Other White	277	9.8

Population	N	%
Asian (ASI)	790	16.9
Asian	232	29.4
Asian- Indian	154	19.5
Asian- Pakistani	150	19
Asian- Bangladeshi	11	1.4
Indian Subcontinent	126	15.9
Other Asian Background	117	14.8
South and East Asian (SEA)	47	1
South East Asian	11	23.4
Chinese	19	40.4
Japanese	6	12.8
Oriental	11	23.4
Black (BLK)	256	5.5
Black	41	16
African Black	144	56.3
Caribbean Black	48	18.8
USA Black	1	0.4
Other Black	22	8.6
Mixed (MIX)	605	13
Mixed	241	39.8
Mixed White and Asian	101	16.7
Mixed White and Black Caribbean	74	12.2
Mixed White and Black African	58	9.6
Other Mixed Background	131	21.7
Other ethnicity (OE)	83	1.8
Native America/Australia/New Zealand	14	16.9
South America	21	25.3
Other	48	57.8
Unknown (UNK)	59	1.3
Unknown/Not disclosed	59	-

3.3.2 Addition of NIMA to inherited CBU HLA A~B~DRB1 haplotypes

An identical-by-descent (IBD) algorithm that compared the maternal and CBU HLA types was developed to identify the CBU IMA/IPA and NIMA HLA A~B~DRB1 haplotypes at first field resolution (as described in **M&M section 2.8.1.1**). HLA A~B~DRB1 haplotypes were identified only for cases whereby the mother and CBU were mismatched at each of the three HLA loci (74.7%, *n*= 3,491). The number of unique CBU IMA/IPA and NIMA HLA A~B~DRB1 haplotypes were identified, by running queries in MS Access, to give an indication of the diversity present amongst the CBUs with maternal HLA types. Results are shown in **Figure 3.2**. There were 1,702 unique CBU IMA/IPA HLA haplotypes from the total 6,982 (2n) and almost half (*n*= 798, 46.9%) of these were only observed once in the cohort **(Figure 3.2A)**. There were 1,243 unique NIMA HLA haplotypes and these were added to the 1,702 CBU IMA/IPA haplotypes to form a combined dataset. HLA A~B~DRB1 haplotypes common to both groups were identified (*n*= 884, 42.8%). Importantly, the inclusion of NIMA identified HLA haplotypes that were not present amongst the CBU IMA/IPA haplotypes (*n*= 361, 17.5%) and these increased the number of unique HLA A~B~DRB1 haplotypes from 1,702 to 2,065 (1.2-fold) in the combined IMA/IPA/NIMA dataset. Of these, 859 (50.5%) HLA A~B~DRB1 haplotypes were only observed once, demonstrating a large diversity.

Figure 3.2 Unique CBU IMA/IPA and NIMA first field HLA A~B~DRB1 haplotypes in the (A) Total, (B) CAU, (C) ASI, (D) SEA, (E) BLK and (F) MIX populations. Maternal and CBU HLA types were compared in an identical-by-descent algorithm to identify allele segregation and haplotypes. Unique IMA/IPA haplotypes were combined with unique NIMA haplotypes to identify haplotypes common to both groups and those that were limited to either group. *Total also includes OE and UNK.

(A) Total* n= 3,491
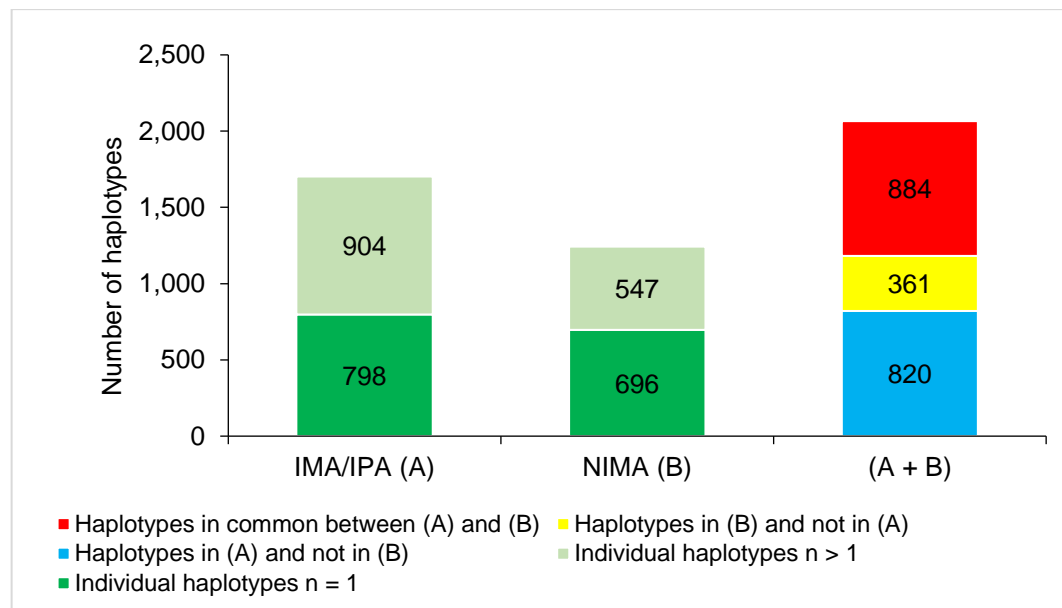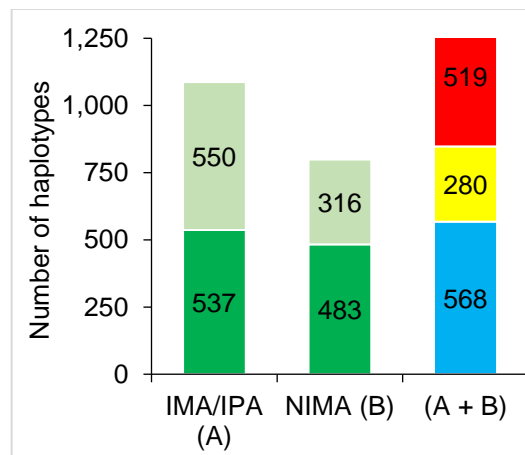
(B) CAU n= 2,093   **(C) ASI** n= 580
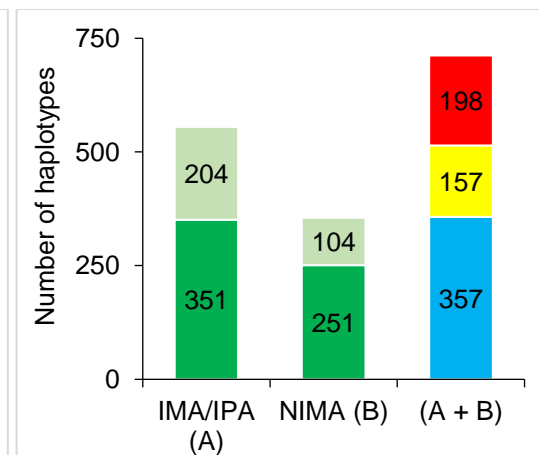
Figure 3.2 continued

(D) SEA *n*= 32
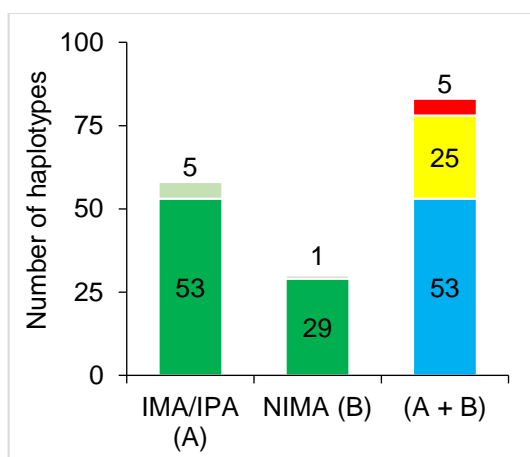
(E) BLK *n*= 202
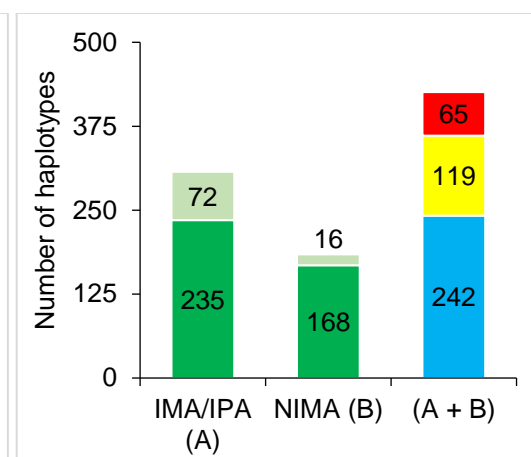
(F) MIX *n*= 471
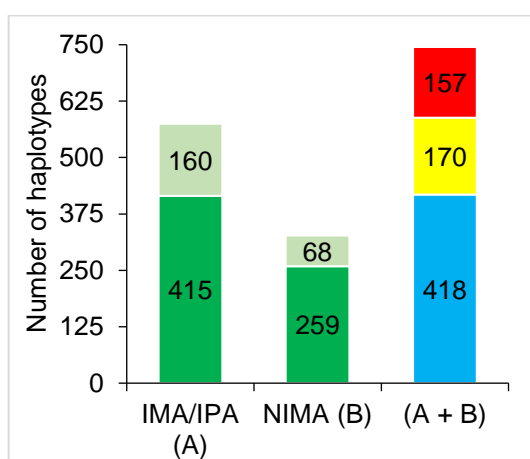
NIMA provided a comparable increase in the number of unique HLA A~B~DRB1 haplotypes in each of the broad populations, despite differences in sample size **(Figure 3.2B-F)**. The number of unique HLA A~B~DRB1 haplotypes increased in the CAU population from 1,087 IMA/IPA to 1,367 IMA/IPA/NIMA (1.3-fold), in the ASI population from 555 to 712 (1.3-fold), in the SEA population from 58 to 83 (1.4-fold), in the BLK population from 307 to 426 (1.4-fold) and in the MIX population from 575 to 745 (1.3-fold). A greater number of IMA/IPA and NIMA haplotypes were only observed once in the non-CAU populations (ASI *n*= 416, 58.4%; SEA *n*= 74, 89.2%; BLK *n*= 306, 71.8%; MIX *n*= 490, 65.8%) compared to

CAU (*n*= 652, 47.7%) (*p*<0.001), likely due to the smaller sample size of these groups.

An exploration of the CBU and maternal HLA allele and A~B~DRB1 haplotype frequencies are given in more detail in **Appendix A**. These were generally comparable to the top HLA A~B~DRB1 haplotype frequencies in the NMDP registry, except for the BLK population (Gragert, *et al* 2013). This was possibly due to the heterogeneity or differences in the American and British BLK populations. Rare HLA haplotypes that ranked in the bottom 25th centile of the corresponding broad populations in the NMDP registry, or were absent in the reported NMDP haplotype frequencies altogether, were also identified.

3.4 Informative and non-informative NIMA at HLA-A, -B and –DRB1

The degree of CBU-maternal MM at *HLA-A*, *-B* and *–DRB1* is shown in **Table 3.2**. The incidence of a CBU-maternal 0 MM was comparable at *HLA-A* (*n*= 517, 11.1%), *-B* (*n*= 359, 7.7%) and *–DRB1* (*n*= 502, 10.7%) (*p*= 0.717), despite the more polymorphic nature of *HLA-B*. However, when stratified by population, significant differences were observed between populations at *HLA-B* (*p*= 0.01). The highest incidence of CBU-maternal 0 MM at *HLA-B* was observed in the ASI population (*n*= 81, 10.3%) and the lowest observed in the MIX population (*n*= 35, 5.8%). No significant difference was observed between broad populations at *HLA-A* (*p*= 0.155) and *–DRB1* (*p*= 0.068).

Table 3.2 Maternal homozygosity and histocompatibility to the CBU at first field *HLA-A*, *-B* and *–DRB1*. Chi-squared (*X²*) analyses: *d.f.* = 6 for individual loci and *d.f.* = 9 for totals. Significant values are shown in bold. Percentages are given in parenthesis. *SEA were not included in the statistical analysis due to the small sample size.

	Total	Population					Statistics*	
		CAU	ASI	SEA	BLK	MIX	X^2	P
CBU-Maternal 0 MM								
HLA-A	517 (11.1)	328 (11.6)	74 (9.4)	9 (19.1)	21 (8.2)	68 (11.2)	5.235	0.155
HLA-B	359 (7.7)	218 (7.7)	81 (10.3)	3 (6.4)	15 (5.9)	35 (5.8)	**11.422**	**0.01**
HLA-DRB1	502 (10.7)	308 (10.9)	99 (12.5)	7 (14.9)	29 (11.3)	49 (8.1)	7.118	0.068
Total MM at *HLA-A*, *-B* and *–DRB1*							11.835	0.223
0	33 (0.7)	18 (0.6)	10 (1.3)	1 (2.1)	2 (0.8)	2 (0.3)	-	-
1	132 (2.8)	80 (2.8)	24 (3.0)	2 (4.3)	7 (2.7)	14 (2.3)	-	-
2	1,015 (21.7)	640 (22.6)	176 (22.3)	12 (25.5)	45 (17.6)	118 (19.5)	-	-
3	3,491 (74.7)	2,093 (73.9)	580 (73.4)	32 (68.1)	202 (78.9)	471 (77.9)	-	-
Maternal homozygosity								
HLA-A	720 (15.4)	473 (16.7)	109 (13.8)	10 (21.3)	25 (9.8)	81 (13.4)	11.055	**0.011**
HLA-B	413 (8.8)	248 (8.8)	84 (10.6)	4 (8.5)	23 (9.0)	39 (6.4)	7.506	0.057
HLA-DRB1	615 (13.2)	371 (13.1)	126 (15.9)	7 (14.9)	34 (13.2)	64 (10.6)	8.800	**0.032**
Total homozygous loci							17.559	**0.041**
0	3,249 (69.6)	1,940 (68.5)	542 (68.6)	29 (61.7)	188 (73.4)	446 (73.7)	-	-
1	1,148 (24.6)	723 (25.5)	191 (24.2)	15 (31.9)	52 (20.3)	137 (22.6)	-	-
2	222 (4.8)	135 (4.8)	43 (5.4)	3 (6.4)	15 (5.9)	19 (3.1)	-	-
3	52 (1.1)	33 (1.2)	14 (1.8)	0	1 (0.4)	3 (0.5)	-	-
Total	**4,671**	**2,831**	**790**	**47**	**256**	**605**		

The majority of CBUs and mothers had 1 MM at *HLA-A*, *-B* and *–DRB1* (1-1-1) and 3 MM in total (*n*= 3,491, 74.7%). Zero MM at only one HLA locus and 2 MM overall (1-1-0, 1-0-1 or 0-1-1) was also frequent (*n*= 1,015, 21.7%). Zero MM at two HLA loci (1-0-0, 0-1-0 or 0-0-1) (*n*= 132, 2.8%) and three HLA loci (0-0-0) (*n*= 33, 0.7%) occurred significantly less frequently (*p*< 0.00001). No significant difference in the sum of MMs across the three HLA loci was observed between the broad population groups (*p*= 0.223).

In the total population, incidences of maternal homozygosity were significantly lower at *HLA-B* (*n*= 413, 8.8%) when compared to *HLA-A* (*n*= 720, 15.4%) and *HLA-DRB1* (*n*= 615, 13.2%) (*p*< 0.00001). Statistical differences in the incidence of maternal homozygosity between the broad populations at *HLA-A* (*p*=0.011) and *HLA-DRB1* (*p*=0.032) were also observed and a trend towards significance was observed at *HLA-B* (*p*=0.057). Maternal homozygosity at *HLA-A* was lower in the BLK population (*n*= 25, 9.8%) compared to CAU (*n*= 473, 16.7%) and homozygosity at *HLA-DRB1* was lower in the MIX population (*n*= 64, 10.6%) compared to ASI (*n*= 126, 15.9%).

The total number of homozygous HLA loci across *HLA-A*, *-B* and *–DRB1* was also determined. In the total population, the majority of mothers were heterozygous across *HLA-A, -B* and *–DRB1* (*n*= 3,249, 69.6%). A quarter of mothers were homozygous at one HLA locus (*n*= 1,148, 24.6%), 4.8% (*n*= 222) at two loci and 1.1% (*n*= 52) at three loci. The total number of homozygous loci was significantly lower in the MIX population compared to CAU and ASI populations (*p*= 0.041).

It was reasoned that the incidence of CBU-maternal 0 MM may be a consequence of probability of two alleles occurring at an HLA locus due to the frequencies of these alleles. The frequencies of first and second position (*x, y* e.g. *A*01*, *A*02* where *A*01* is *x* and *A*02* is *y*) allele combinations were therefore determined by a direct count method. To determine if 0 MM was more likely for high frequency allele combinations, these were ranked in order of frequency and then split into cumulative 25% quartiles (Q1: bottom 25%, Q2: 25-50%, Q3: 50-75%, Q4: 75-100%) and comparisons were made between the quartiles. Results are shown in **Figure 3.3** for **(A)** *HLA-A*, **(B)** *HLA-B* and **(C)** *HLA-DRB1*. The incidence of 0 MM between a mother and CBU was highly associated with the frequency of the allele combination in the total population for *HLA-A*, *-B* and *-DRB1*. Allele combinations at an HLA locus in the upper quartile (i.e. occurred more frequently in the total population) were more likely to be identified as a 0 MM between the mother and CBU for *HLA-A* ($p<0.0001$), *HLA-B* ($p<0.0001$) and *HLA-DRB1* ($p<0.001$). When further stratified by ethnicity, CBU-maternal 0 MM was significantly associated with the frequency of an allele combination at *HLA-A* across all ethnic groups, except for the smaller population size of SEA. However, at *HLA-B* and *–DRB1*, CBU-maternal 0 MM was only significantly associated with the frequency of an allele combination in the CAU population. The top 10 ranking *x, y* allele combinations at *HLA-A*, *-B* and *-DRB1* and the proportion of these that were a CBU-maternal 0 MM in each of the populations are given in **Appendix A.** The heterozygous allele combinations with the highest frequency in the total CBU population were as follows for *HLA-A*, *-B* and *-DRB1*: *A*01, A*02* (9.7%, *n*= 392/4,042) and this was a 0 MM to the mother in 20.2% of cases (*n*= 79/392); *B*07, B*44* (3.3%, *n*= 139/4,269) and this was a 0 MM to the mother in 10.8% of cases (*n*= 15/139) and

DRB1*04, *DRB1*15* (5.3%, *n*= 218/4,076) and this was a 0 MM to the mother in

15.1% of cases (*n*= 33/218).

Figure 3.3 Percentage of heterozygous CBU-Maternal 0 MM according to *x, y* allele frequencies at (A) *HLA-A*, (B) *-B* and (C) *–DRB1*. Frequencies of individual heterozygous *x, y* allele combinations were calculated by a direct count. The cumulative frequencies of the highest to lowest ranking were used to split the data into quartiles (Q). Q1: bottom 25%, Q2: 25-50%, Q3: 50-75%, Q4: 75-100%. Results show the percent of 0 MM out of the total *x, y* alleles in each quartile. Total also includes OTH and UNK categories. * *P* <0.05, ** *P* <0.01, *** *P* <0.001, **** *P* <0.0001.

(A) *HLA-A*
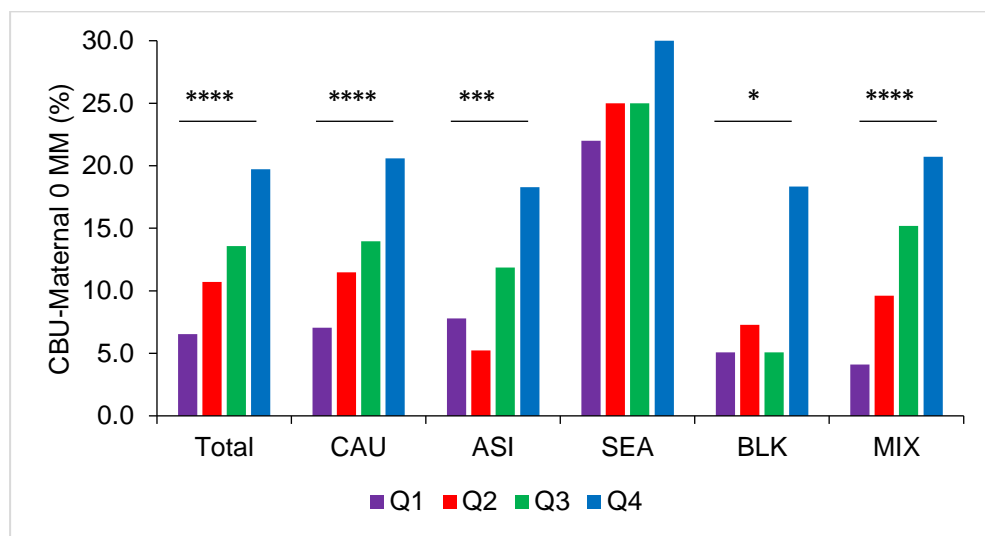
(B) *HLA-B*
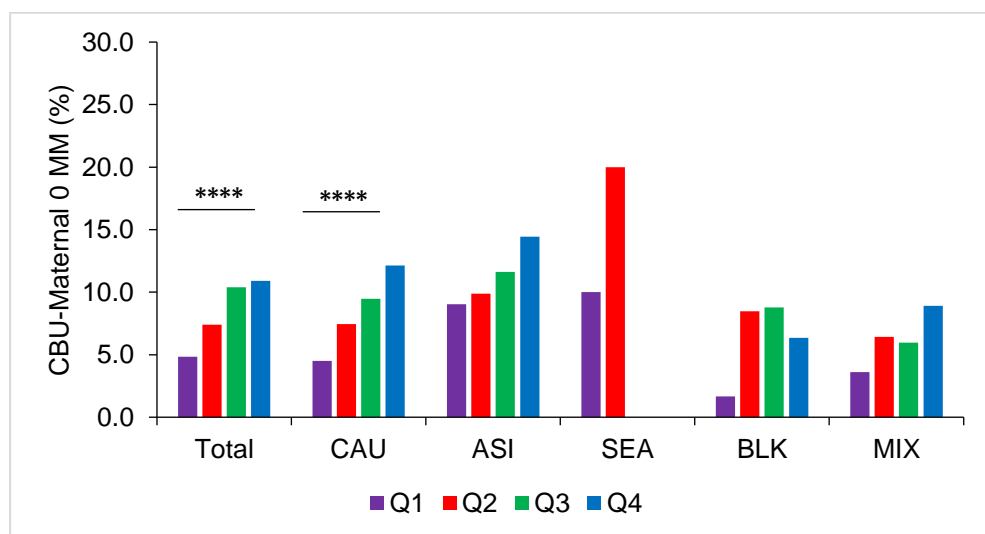
Figure 3.3 continued

(C) *HLA-DRB1*
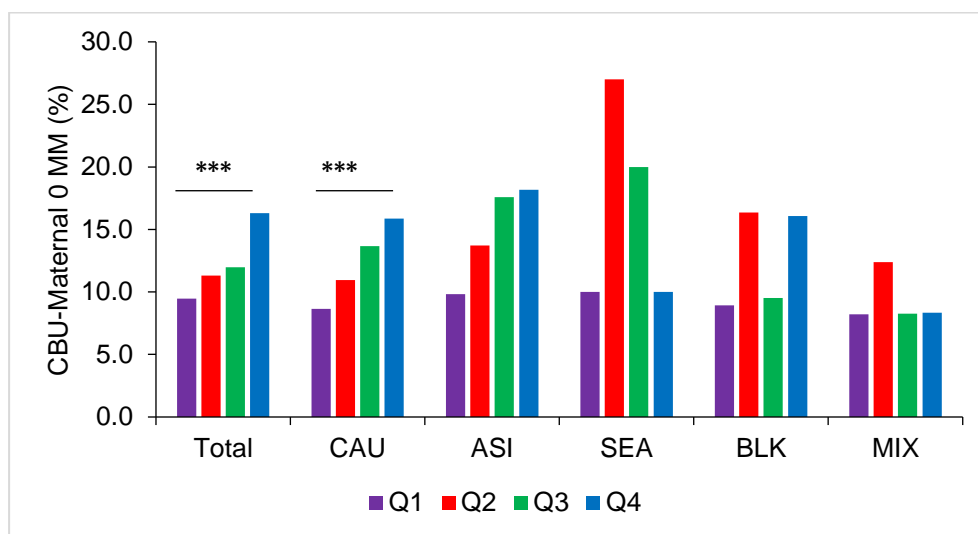
Maternal homozygosity and histocompatibility to the CBU were used to identify the number of CBUs informative for 0, 1, 2 or 3 NIMA at *HLA-A, -B* and *–DRB1* and results are given in **Figure 3.4**. In the total population, 4,535 (97.1%) CBUs were informative for 1 - 3 NIMA. There were 2,307 (49.4%) CBUs informative for NIMA at 3 HLA loci, 1,742 (37.3%) informative at 2 loci and 486 (10.4%) informative at 1 locus. The remaining 136 (2.9%) CBUs were not informative for NIMA at any of the three HLA loci. Significant differences in the total number of informative NIMA were observed between the broad populations (*p*< 0.0001). A greater proportion of BLK (55.5%) and MIX (54.9%) CBUs were informative for 3 NIMA compared to CAU (47.5%) and ASI (49.2%). The inverse was observed for the proportion of CBUs informative for 0 NIMA.

Figure 3.4 CBUs informative for 0 – 3 NIMA at *HLA-A*, *-B* and *–DRB1*. NIMA were informative to first field at an HLA locus if the mother was heterozygous and mismatched to the CBU. *Total also includes SEA (*n*= 47), OE (*n*= 83) and UNK (*n*= 59) CBUs. **** *p* < 0.0001.

	Total	CAU	ASI	BLK	MIX
Total	4,671	2,831	790	256	605
■ 0 NIMA	136	83	38	4	8
■ 1 NIMA	486	294	96	28	46
■ 2 NIMA	1,742	1,107	267	81	219
■ 3 NIMA	2,307	1,347	389	143	332
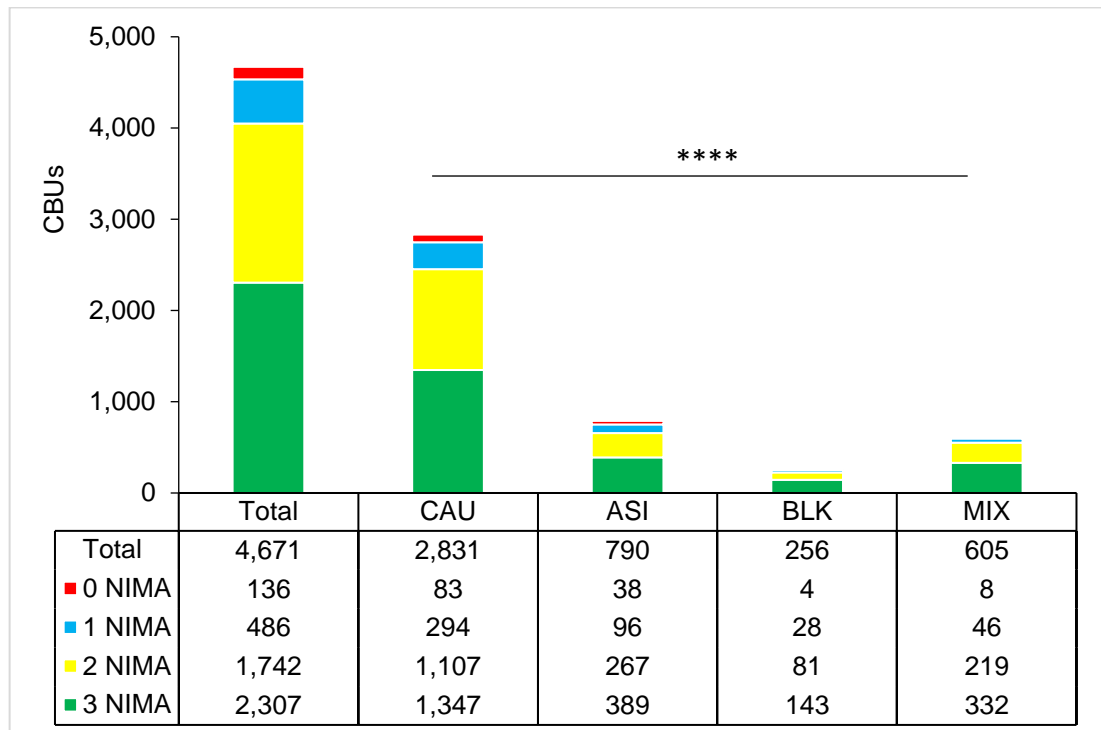
In summary, incidences of non-informative NIMA at each HLA locus and due to 0 MM between the mother and CBU (7 – 11%) or maternal homozygosity (8 – 15%) were comparable. There were no statistically significant differences between the HLA loci, except maternal homozygosity was significantly higher at *HLA-B* compared to *HLA-A* and *–DRB1*. When comparisons were made between populations, CAU and ASI populations were less likely to be informative for NIMA compared to BLK and MIX populations.

3.5 Generation of VPs and the subsequent increase to the pool of phenotypes in the NHS-CBB

3.5.1 Quantification of VPs

The number of VPs provided by HLA typing 4,671 mothers of CBUs compared to the maximum expected (ME) if all CBUs were informative for NIMA at *HLA-A, -B* and *–DRB1* is shown in **Figure 3.5.** The ME number of VPs from 4,671 CBUs if all were informative for 3 NIMA was 121,446 (26-fold increase). The actual observed fold increase was lower - 14.2-fold, giving a total of 66,225 VPs. In accordance with the differences observed in the number of informative NIMA between the broad populations described in the previous section, some differences were also observed in the number of VPs generated. Both the CAU and ASI populations yielded a comparable fold increase in VPs (13.9 fold and 13.7 fold, respectively). The 2,831 CAU CBUs yielded 39,283 VPs and the 790 ASI CBUs yielded 10,860 VPs. Both the BLK and MIX populations showed a higher fold-increase (15.6 and 15.5 fold, respectively) in the number of VPs because these populations were more likely to be informative for NIMA. The 256 BLK CBUs yielded 3,987 VPs and the 605 MIX CBUs yielded 9,406 VPs.

Figure 3.5 Maximum expected versus observed number of VPs. VPs were generated from an in-house algorithm by substituting 1 – 3 of the CBU HLA for the corresponding NIMA at *HLA-A, -B* and/or *–DRB1*. A substitution at an HLA locus is only made if the NIMA is informative (mother is heterozygous and mismatched to the CBU). The maximum expected number of VPs is based on the generation of 26 per CBU if the 3 loci were each informative for NIMA. Total also includes SEA, OE and UNK.
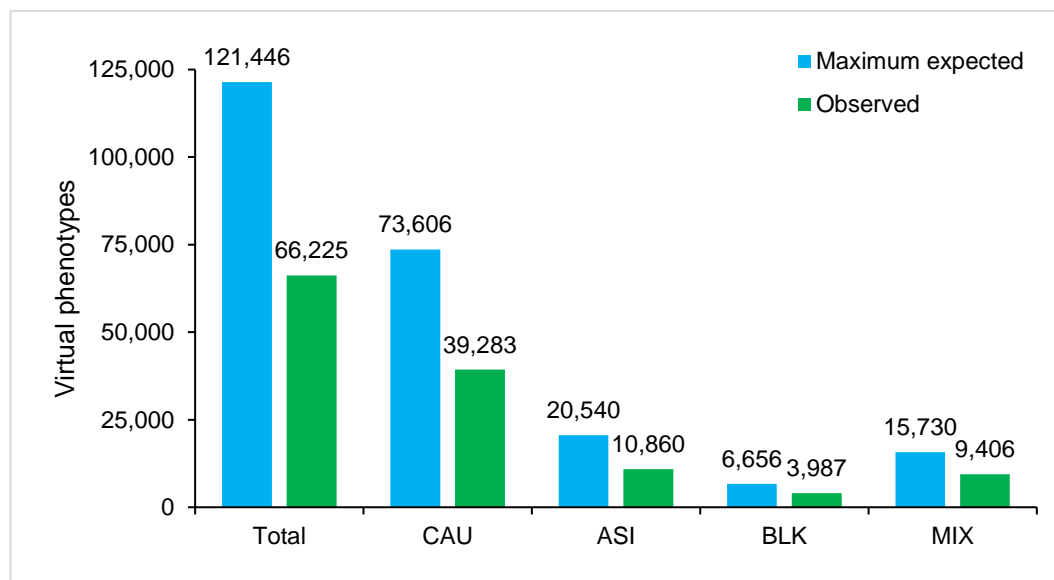
3.5.2 Representation of new phenotypes amongst CBUs with maternal HLA typing from the generation of VPs

VPs were next compared to the existing inherited phenotypes (IPs) of the 4,671 CBUs with maternal HLA **(Figure 3.6A–E)**. There were 4,304 unique IPs at first field *HLA-A, -B* and *-DRB1* from the total 4,671 CBUs (A). There were 66,225 VPs generated from these and 52,875 were unique VPs (B). When the IPs and VPs were combined to form a single dataset, the number of unique phenotypes increased 13-fold from 4,304 to 55,783 unique phenotypes (A + B). There were 1,396 (2.5%) phenotypes represented as both IPs and VPs. There were 2,908 (5.2%) represented as only an IP. The remaining 51,479 (92.3%) were new VPs

that were not yet represented as IPs amongst the 4,671 CBUs with maternal HLA

in the NHS-CBB.

Figure 3.6 Addition of virtual phenotypes (VPs) to the inherited phenotypes (IPs) of CBUs with maternal HLA typing. (A) Total* *n*= 4,671 **(B) CAU** *n*= 2,831 **(C) ASI** *n*= 790 **(D) BLK** *n*= 256 and **(E) MIX** *n*= 605. HLA phenotypes refer to first field HLA-A, -B and -DRB1. Unique phenotypes are the total phenotypes with duplicates removed. VPs have 1 – 3 substitutions of the CBU inherited HLA for the corresponding NIMA at *HLA-A, -B* and/or *–DRB1* and were generated from an in-house algorithm. IPs are the CBU phenotypes from the inherited maternal and paternal haplotype and without substituting for NIMA. Phenotypes common or unique to the inherited (A) and virtual (B) phenotypes were identified by running queries in MS Access. Total VPs (includes duplicates): Total, 66,225; CAU, 39,283; ASI, 10,860; BLK, 3,987; MIX, 9,406. *Includes SEA, OE and UNK CBUs and mothers.

(A) Total

	Inherited (A)	Virtual (B)	(A) + (B)
■ Unique phenotypes	4,304	52,875	55,783
■ Common between (A) and (B)			1,396
■ Present in (A) but not (B)			2,908
■ Present in (B) but not (A)			51,479
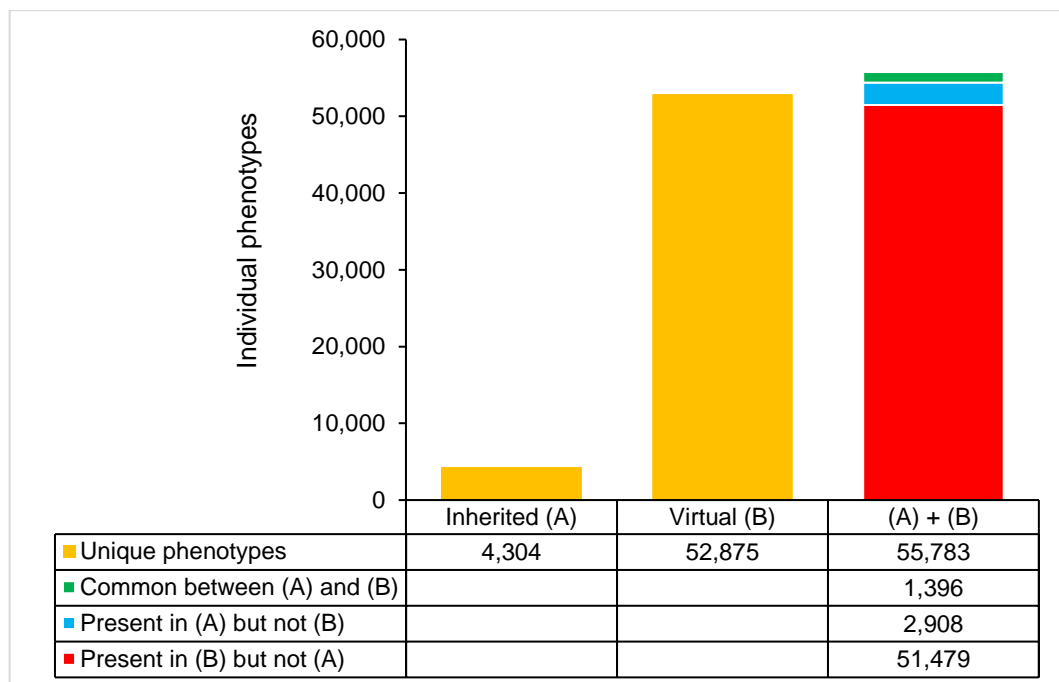
Figure 3.6 continued

(B) CAU

	Inherited (A)	Virtual (B)	(A) + (B)
🟨	2,569	31,094	32,794
🟩			869
🟦			1,700
🟥			30,225

(C) ASI

	Inherited (A)	Virtual (B)	(A) + (B)
🟨	777	10,066	10,729
🟩			114
🟦			663
🟥			9,952

(D) BLK

	Inherited (A)	Virtual (B)	(A) + (B)
🟨	255	3,900	4,148
🟩			7
🟦			248
🟥			3,893

(E) MIX

	Inherited (A)	Virtual (B)	(A) + (B)
🟨	599	9,201	9,747
🟩			53
🟦			546
🟥			9,148
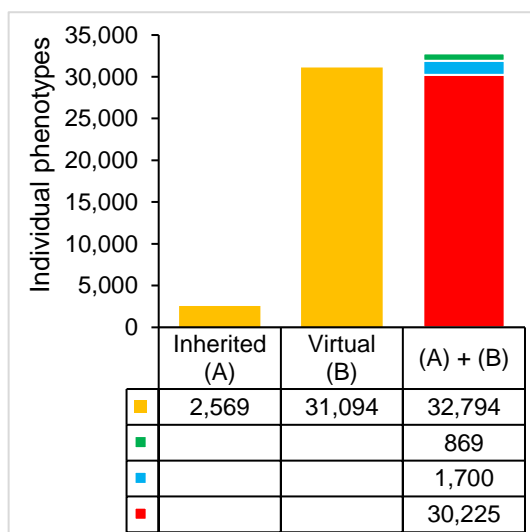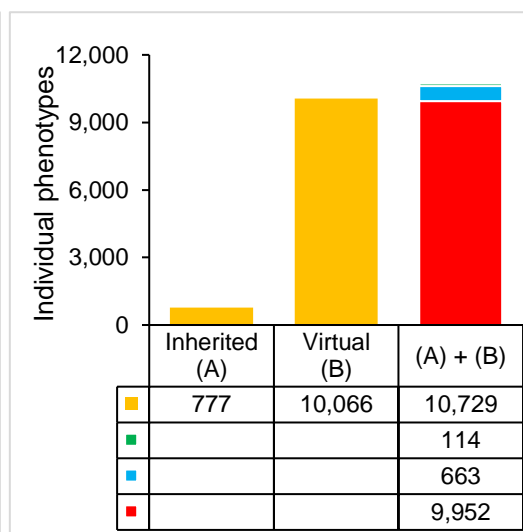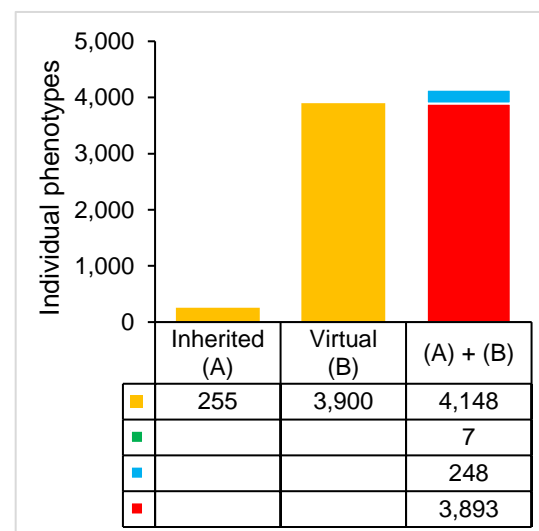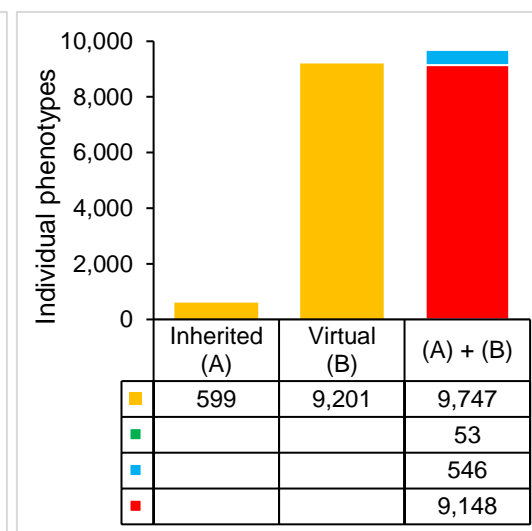
Comparisons were made between the broad populations **(Figure 3.6B–E)**. The increase in the number of unique phenotypes was comparable between the CAU and ASI populations: 12.8-fold from 2,569 IPs to 32,794 unique IPs plus VPs (IPs+VPs) in the CAU population and 13.8-fold from 777 IPs to 10,729 unique IPs+VPs in the ASI population. There was a greater increase in the number of unique phenotypes in the BLK and MIX population: 16.3-fold from 255 IPs to

4,148 unique IPs+VPs in the BLK population and 16.3-fold from 599 IPs to 9,747 unique IPs+VPs in the MIX population.

3.5.3 Representation of new phenotypes to the NHS-CBB from the generation of VPs

It was next determined whether the 66,225 VPs provided from the 4,671 CBUs with maternal HLA represented new phenotypes that were not yet present as IPs from the total 21,020 banked CBUs in the NHS-CBB **(Figure 3.7).** The NHS-CBB CBUs showed a diverse range of inherited first field *HLA-A*, *-B* and *-DRB1* phenotypes, with 16,825 (80.0%) unique IPs from the total 21,020 CBUs (A). The VPs also showed little duplication, with 52,875 (79.8%) unique phenotypes (B) from the total 66,225 VPs. The unique IPs and unique VPs were added together to create a combined pool of phenotypes (A+B, IPs+VPs) available for both inherited and virtual matched donor searches. Maternal HLA typing just a quarter of the CBUs in the NHS-CBB increased the number of unique phenotypes present by 3.9 fold to 65,046 (A + B). There were 4,654 (7.2%) phenotypes present in both the inherited and VPs, demonstrating that these VPs also exist as IPs in the NHS-CBB donor population. There were 12,171 (18.7%) that were only present in the IPs. The remaining 48,221 (74.1%) were new VPs that were not yet represented as IPs in the NHS-CBB.

Figure 3.7 Addition of virtual phenotypes (VPs) to the inherited phenotypes (IPs) of the entire NHS-CBB. HLA phenotypes refer to first field HLA-A, -B and -DRB1. Unique phenotypes are the total phenotypes with duplicates removed. VPs have 1 – 3 substitutions of the CBU inherited HLA for the corresponding NIMA at *HLA-A, -B* and/or *–DRB1* and were generated from an in-house algorithm. IPs are the CBU phenotypes from the inherited maternal and paternal haplotype and without substituting for NIMA. Phenotypes common or unique to the inherited (A) and virtual (B) phenotypes were identified by running queries in MS Access. Unique IPs: 16,825 out of 21,020 CBUs. Unique VPs: 52,875 out of 66,225 VPs generated from 4,671 CBUs.

	Inherited phenotypes n= 21,020 CBUs (A)	Virtual phenotypes n= 4,671 CBUs (B)	(A) + (B)
■ Unique phenotypes	16,825	52,875	65,046
■ Common between (A) and (B)			4,654
■ Present in (A) but not (B)			12,171
■ Present in (B) but not (A)			48,221
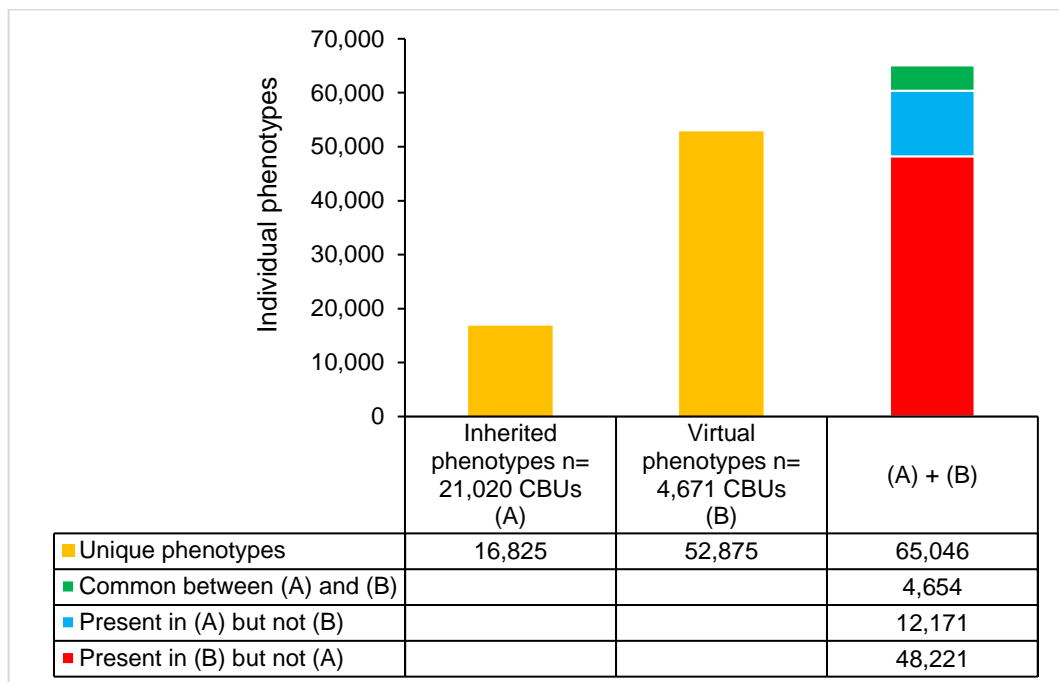
3.6 Discussion

3.6.1 NHS-CBB mothers and their CBUs are diverse in terms of ethnicity and HLA frequencies

The probability of identifying a NIMA match is likely to be aided by knowledge of the HLA allele and haplotype frequencies present in the registry. For patients with only a mismatched donor available, knowledge of NIMA will help facilitate the most appropriate HLA(s) to MM and target as a possible NIMA match. This study describes the first field NIMA HLA allele and A~B~DRB1 haplotype frequencies present in the NHS-CBB. In addition to the identification of the NIMA, the unique opportunity to assign registry HLA haplotypes by the comparison of mother and CBU HLA types meant it was possible to evaluate actual, rather than estimated, HLA haplotype frequencies for 75% (*n*= 3,491/4,671) of CBUs with maternal HLA typing available.

Mathematical models such as E-M are invaluable tools for predicting HLA haplotype phasing but several problems arise when used in registry datasets (Gragert*, et al* 2013, Kollman*, et al* 2007). The frequencies of common HLA haplotypes can be overestimated and rare haplotypes are sometimes systemically excluded from analysis due to the possibility of algorithm artefacts, especially in the context of multiple loci or mixed HLA typing resolution data (Bettencourt*, et al* 2008, Castelli*, et al* 2010). Accurate estimation of HLA haplotypes also assumes the population studied is in Hardy-Weinberg Equilibrium, which is not always true for small sample sizes or if admixture is present (Bettencourt*, et al* 2008). Comparison of mother and child HLA types does not require this assumption and I was able to include mothers and CBUs of

mixed ethnicity, which make up a large proportion of the NHS-CBB. It should be noted that paternal HLA typing data was not available and a small number of HLA haplotypes could have arisen from recombination, although this is generally thought to have a frequency of less than 1% (Lam, *et al* 2013).

The NHS-CBB targets the recruitment of minority ethnic populations and around 40% of banked CBUs are from a non-CAU background, compared to 2% in the adult BBMR (Armitage, *et al* 1999, Davey, *et al* 2004). The small number of CBUs with maternal HLA typing are very heterogeneous, demonstrated by the observation that 1,702 unique first field IMA/IPA HLA A~B~DRB1 haplotypes were identified from 3,491 CBUs, of which 46.9% were only observed once in the cohort. Rare HLA A~B~DRB1 haplotypes were also identified, judged by their absence in the estimated frequencies in the NMDP registry. Continuing to perform maternal HLA typing for additional CBUs is likely to further increase the representation of CBU IMA/IPA and NIMA HLA haplotypes in the NHS-CBB.

3.6.2 The number of informative NIMA differed between broad populations

This study has explored reasons why the NIMA is non-informative and whether any differences were observed between HLA loci and donor populations. One or more NIMA were non-informative to first field at *HLA-A*, *-B* and/or *-DRB1* for half (50.6%, *n*= 2,454/4,761) of CBUs. This was most often because the NIMA was non-informative at just one of the three HLA loci (37.3%, *n*= 1,742/4,671) and it was rare that the CBU was not informative at any of the three HLA loci considered (2.9%, *n*= 136/4,671). This means that in the overwhelming majority of cases,

VPs could still be generated if the maternal HLA type was defined, albeit below the maximum potential of 26 per CBU.

It seems reasonable that the CBU IMA/IPA and maternal NIMA HLA could readily be distinguished given their natural function and that the HLA genes are the most polymorphic region of the human genome. Polymorphisms are likely to have arisen from natural selection and an individual that is heterozygous at an HLA locus is likely to have an advantage, with regards to presentation of peptides from an infectious agent, compared to those that are homozygous (Buhler and Sanchez-Mazas 2011, Sommer 2005). For example, HIV positive patients that are homozygous at one or more class I HLA loci have shown faster progression to AIDs compared to heterozygous patients (Carrington, *et al* 1999). The production of offspring with an HLA heterozygous advantage may be enforced through mate choice, with several studies suggesting that humans may prefer HLA dissimilar mates, possibly mediated through body odour attraction, although results are conflicting (Chaix, *et al* 2008, Wedekind, *et al* 1995, Winternitz, *et al* 2016).

Significant differences in the incidences of informative and non-informative NIMA were identified between the different broad populations. Non-CAU donors and their phenotypes are exceptionally valuable with regards to NHS-CBB diversity and the aim to improve donor availability for ethnic minorities requiring HSCT. Importantly, the total number of informative NIMA and generation of VPs was not lower for non-CAU populations and rather, BLK and MIX populations were more likely to be informative for NIMA. This most probably a reflection of the multiple ethnic background of MIX CBUs and extensive genetic diversity within the African population (Cao, *et al* 2004, Testi, *et al* 2015) but

contrasts a previous analysis that found European American couples from the Mormon community exhibited HLA dissimilarity compared to random individuals (controls) but African couples did not (Chaix, *et al* 2008). As a consequence to the incidence of informative NIMA, the fold-increase in phenotypes observed was actually higher in the BLK and MIX populations (15.6) compared to CAU (13.9) and ASI (13.7), suggesting a greater yield to the bank in maternal HLA typing these CBUs. Donor searches of VPs may be particularly beneficial to BLK patients requiring CBT, for whom donor availability is considerably lower (Gragert, *et al* 2014).

The reasons why there were differences between the broad populations in informative and non-informative NIMA are unclear. It may be due to differences in population genetics and heterogeneity amongst the populations. At *HLA-A*, the incidence of 0 MM between the CBU and mother did not differ between populations. However, the incidence of 0 MM at *HLA-A* was significantly associated with allele frequencies, which suggests the occurrence of 0 MM was due to the mathematical chance based on population frequencies. Alternatively, the differences in broad populations may be due to social, cultural or biological differences. At *HLA-B*, allele frequencies were not associated with the incidence of CBU-maternal 0 MM in non-CAU populations, suggesting there may be additional factors other than chance affecting histocompatibility in these populations. However, the highly selective population grouping for this thesis may not be reflective of mating groups. The small sample size of non-CAU populations could have also meant the reported allele frequencies were not reflective of the general population and indeed, BLK allele and haplotype

frequencies were very different to those reported for the NMDP (see **Appendix A**).

3.6.3 HLA typing 4,671 mothers of CBUs yielded 66,225 VPs and increased the pool of unique phenotypes in the NHS-CBB four-fold

At the start of this study, the number of VPs present in the NHS-CBB was unknown. Although NIMA substitutions could potentially provide up to 26 VPs per CBU, the actual increase from 4,671 CBUs was fourteen-fold, to 66,225 VPs, which confirms the findings of a previous study (Van der Zanden, *et al* 2014) and for the first time, identifies differences between populations. The vast majority of VPs were unique phenotypes and were not yet present amongst the IPs of the NHS-CBB.

The 4,654 VPs that were also present as IPs in the NHS-CBB demonstrate that these can exist as true phenotypes in the donor population. However, the considerable proportion of VPs that were not represented as IPs in the NHS-CBB warrant further investigation. Two possibilities exist, either they are not present as genuine phenotypes in the population and thus will never be utilised as a virtual match for a patient, or they do exist as phenotypes but these are not present in the NHS-CBB and thus could be identified as a virtual match for a patient with such a phenotype. Given that the number of unique VPs (52,875) is far greater than the number of unique IPs (16,825) amongst the CBUs in the NHS-CBB, it seems sensible to determine the proportion of VPs represented as IPs in a much larger bank/registry, such as BMDW, which currently has over 29 million donors registered.

It seems likely that not all VPs will be represented as IPs in BMDW (especially given the results of the next chapter) and whether VPs are likely to exist as true IPs in the population will require novel methods of analyses. This could include testing whether the VPs could theoretically consist as two HLA blocks that are known to exist as two haplotypes from previous registry estimations of haplotype frequencies. The E-M algorithm cannot be used to estimate artificial phasing of the VPs because this method identifies common patterns of alleles within the dataset and these patterns would not be from actual IPs within a population. A more appropriate method would be to develop a tool that identifies all the possible combinations of alleles within a VP and checks if any of these have been reported as haplotypes in the population.

Estimating the artificial phasing of VPs (i.e. possible haplotype combinations) would also permit analyses of whether HLA-B/C combinations of VPs differ to those normally found in LD. The chance of identifying an HLA matched donor is not just hindered by the presence of a rare patient HLA allele - patients with unusual combinations of frequent HLA alleles (i.e. a rare phenotype) will also have a low probability of finding a match (Tiercy 2012). It is not known whether there are any potential benefits of NHS-CBB VPs to donor availability for patients with unusual associations, although it seems likely based on a previous analysis. Van der Zanden and colleagues used a computer model to assign haplotype frequency-based simulated VPs for 167,201 CBUs and predicted that a virtual match would be found for 42 out of 103 patients for whom no donor was available (9/10 or 10/10 MUD or 6/6 CBU) (Van der Zanden, *et al* 2014). Similar analyses of donor availability from the NHS-CBB VP pool for patients with rare

phenotypes, or indeed, patients in general, would be a valuable tool in assessing the benefit to patients by performing maternal HLA typing of banked CBUs.

3.6.4 Limitations

This study included only first field resolution *HLA-A*, *-B* and *–DRB1* in the description of HLA allele and haplotype frequencies and calculation of the VPs present in the NHS-CBB due to the presence of heterogeneous HLA typing resolution data. Distinct serological specificities encoded by alleles with the same first field molecular type were not distinguished from one another, yet donor-recipient matching at this level is clinically relevant (Eapen*, et al* 2014). Antigens B62, B63, B75, B76 and B77 are protein products of *HLA-B*15*, encoded by different alleles. For example, the *B*15:01* gene product is B62 and the *B*15:02* gene product is B75. These differ in population distribution patterns and are associated with different haplotypes. *B*15:01* is frequent in CAU populations and is associated with *C*03:03* or *C*03:04* (encoding Cw9 and Cw10, respectively), whereas *B*15:02* is frequent in ASI populations and is associated with *C*08:01* (Gragert*, et al* 2013).

Inclusion of second field HLA types will yield a greater number of unique VPs because there will be fewer duplicates. It is plausible the second field resolution will further increase the proportion of mothers and CBUs whereby complete NIMA haplotypes can be identified because if there is a 0 MM at an HLA locus between the mother and CBU at first field, there may be differences at second field and thus the NIMA could be identified. For example, if both the mother and CBU are *A*01, 02* but second field resolution reveals the mother as *A*01:01, 02:01*

and the CBU as *A*01:01, 02:07* then *A*01:01* can be identified as the IMA and the maternal *A*02:01* as the NIMA. The Singapore CBB has previously reported the ability to assign four loci haplotypes to second field resolution for 72% of 174 mothers and CBUs (Tang*, et al* 2007) and a study of African mothers and CBUs reported second field five loci haplotypes for 69% of 374 mothers and CBUs (Tu*, et al* 2013). These are comparable to the identification of three loci first field haplotypes for 75% of mothers and CBUs in this study, despite the inclusion of additional HLA loci, which was possibly compensated by the higher HLA typing resolution evaluated. The advent of next-generation HLA typing technology will likely improve the availability of unambiguous five or six loci HLA data for CBUs and mothers.

3.6.5 Conclusions

Performing maternal HLA typing on banked CBUs provides a substantial increase in the number and diversity of phenotypes available for donor searches, without the financial cost of recruiting, processing, testing and banking of additional CBUs. The next chapter will explore whether the VPs in the NHS-CBB are able to provide a virtual 6/6 HLA match for patients.

Chapter 4 NIMA as permissible HLA MMs in cord blood donor selection for patients of various ethnicities

Key points:

- A virtual full match (5/6 + 1 NIMA, 4/6 +2 NIMA or 3/6 + 3 NIMA) was identified for 28.4% and 48.4% of 457 patients by searching the VPs in the BBMR and BMDW registries respectively.
- Virtual full matches provided a greater benefit to other ethnicity patients compared to European Caucasoid patients due to the lower availability of an inherited full match but a comparable availability of a virtual full match for other ethnicity patients.

Publications associated with this chapter:

Powley, L., Brown, C., Melis, A., Li, Y., Parkes, G. & Navarrete, C.V. (2016) Consideration of noninherited maternal Ags as permissible HLA mismatches in cord blood donor selection. *Bone Marrow Transplant,* **51,** 675-679.

4.1 Introduction

The previous chapter showed that the identification of the CB NIMA from maternal HLA typing provided a substantial increase in the number of phenotypes present in the NHS-CBB from the generation of VPs. Crucially, a large proportion of these VPs were new phenotypes not yet represented as inherited phenotypes in the NHS-CBB. However, it is not yet clear whether these VPs are able to provide a virtual full match (VFM, 5/6 + 1 NIMA, 4/6 + 2 NIMA or 3/6 + 3 NIMA) for patients requiring CBT.

A previous study evaluating NIMA match likelihoods identified VFMs for 32% of 2,020 Dutch patients (Van der Zanden, *et al* 2014). This study demonstrated for the first time that VFMs could indeed be identified for patients. However, it did not address two significant factors in donor availability: patient ethnicity and TNC dose. Inherited HLA matched donor availability is known to differ between patients of various ethnicities due to the HLA frequencies in the patient and donor populations (Gragert, *et al* 2014, Gragert, *et al* 2013) and it seems feasible to extend this to the availability of NIMA matched CB donors. A major limitation of CB as a graft source has been the lower TNC dose, which is associated with a higher risk of graft failure and delayed engraftment, particularly in the context of a poor HLA match (Barker, *et al* 2010b) and thus recommendations exist for the minimum cell dose requirements (Hough, *et al* 2016).

Modelling HLA matched donor availability is important for ensuring that CBBs are able to provide suitable donors for the population that they serve and thus aids the strategic planning of CBBs with regards to size and recruitment

strategies. This is a particularly important for the NHS-CBB because the UK population is ethnically diverse, with 20% of respondents for the 2011 census self-identifying with an ethnic group that was not White British (www.ons.gov.uk). HLA matched donor availability models can also aid with designing a treatment plan for a patient by giving an indication of whether a suitable donor is likely to be found. For example, if an HLA matched CB donor is not identified during the initial search, waiting for a 6/6 HLA match (or indeed an HLA matched adult donor) could cause a delay in proceeding to transplant, during which there is a risk of developing more advanced disease or for malignant diseases, relapsing before a transplant is arranged (Gratwohl, *et al* 2009). Indeed, in adult donor HSCT, if an HLA matched donor is not identified early on during the search process; it is unlikely that a new donor that is a match will be added to the registry in a timely manner. In cases where the transplant is urgent, an HLA MM strategy would be preferred and in CBT, one option is to select a NIMA+ HLA mismatch. Obviously, this also requires knowledge of NIMA match probabilities.

4.2 Aims

The primary aim of this chapter was to determine whether the inclusion of VPs, to identify VFMs, can improve donor availability for patients of various ethnicities requiring CBT. There were 457 patients who were previous recipients of an NHS-CBB CBU and they were treated as newly presenting patients requiring CBT. The existing BMDW NIMA match program was used to manually search for potential VFMs and inherited full matches (IFMs) based on matching at *HLA-A* and *–B* at antigenic resolution and *–DRB1* at high resolution, from the CB donors listed with the BBMR or BMDW.

Several questions were formulated to address the primary aim **(Figure 4.1)**. The first question was "what is the probability of identifying a VFM for patients of various ethnicities requiring CBT?" The VPs generated from the previous chapter were searched to provide a VFM, that was a 5/6 + 1 NIMA, 4/6 + 2 NIMA or 3/6 + 3 NIMA match, for patients of various ethnicities. Given that a greater number of inherited HLA matches are likely to be preferred and that when HLA mismatching, class I MMs tend to be preferred over class II MMs, the locus and total number of NIMA matches (1, 2 or 3) for VFMs were also determined. This led to the second question, "can VFMs provide an alternative for patients of various ethnicities without an IFM?" This required a comparison of the inherited HLA matched donors identified (6/6, 5/6, 4/6 or 3/6) from searches of banked CBUs; to the VFM donors identified by searching the VPs. Given that TNC dose is also crucial for CB donor identification, the third question was "can VFMs, with adequate cell dose, provide an alternative for patients of various ethnicities without an IFM and requiring a single graft?"

Figure 4.1 Chapter 4 aim and the question proposed to address this aim

Aim: To determine if the inclusion of virtual phenotypes, to provide virtual full matches, can improve donor availability for patients of various ethnicities requiring CBT.

What is the probability of identifying a virtual full match for patients of various ethnicities requiring CBT?

Can virtual full matches provide an alternative for patients of various ethnicities without an inherited full match?

- Number of NIMA matches (1, 2 or 3)
- HLA loci of NIMA matches (HLA-A/B/DRB1)
- Patients with rare HLA alleles

- Inherited HLA match grades of CB donors identified.
- Proportion of patients without an inherited full match but with a virtual full match

Can virtual full matches, with adequate cell dose, provide an alternative for patients of various ethnicities without an inherited full match and requiring single CBT?

4.3 Patient demographics

Four hundred and fifty-seven previous recipients of an NHS-CBB issued CBU were treated as newly presenting patients requiring CBT. The median patient age and weight were 19 years and 55 kg, respectively, and 80.7% (n= 369) were treated for malignant indications **(Table 4.1)**. Self-reported ethnicities were available for 341 patients and were mapped to broad population groups. The majority (80%) were European Caucasoid (EC) (n= 274). The remaining 20% included non-EC (n= 35), Black (n= 13), South and East Asian (n= 6) or mixed ethnicity (n= 13) and due to the small cohort size, were grouped as a single category, other ethnicity (OE), for all further analyses.

Table 4.1 Patient demographics. Percentages are given in parenthesis unless otherwise stated.

Demographic	Number of patients
Gender	
Male	274 (60.0)
Age	
≤ 16 years	218 (47.7)
Median age (range)	19 (0 - 70)
Weight (kg)	
<50 kg	204 (44.6)
Median weight (range)	55 (3 - 146)
Ethnicity (unknown/not declared *n*= 116)	
European Caucasoid	274 (80.4)
Other ethnicity	67 (19.6)
– Non-European Caucasoid	35
– South and East Asian	13
– Black	6
– Mixed	13
Disease indication	
Malignant	369 (80.7)
– ALL	93
– AML	132
– MDS or MDS/MPD	81
– Lymphoproliferative disorder	46
– Plasma cell disorder	7
– Histiocytic disorder	9
– Solid tumour	1
Non-malignant	88 (19.3)
– Bone marrow failure syndrome	26
– Haemoglobinopathy	3
– Primary immune deficiency	30
– Inborn error of metabolism	29
Total patients	457

Abbreviations: ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome; MPD, myeloproliferative disorder

4.4 BMDW and BBMR virtual full matches

Donor searches for VFMs were performed using the BMDW NIMA Match Program and by searching donors listed with the BBMR or BMDW (see M&M **section 2.8.2**). The BMDW NIMA Match Program uses an algorithm to generate the VPs of a CB donor (using the same methodology described in the previous chapter) and then uses these VPs to search for a VFM to the patient. A VFM was identified for 28.4% of 457 patients by searching the 4,707 CB donors with maternal HLA listed with the BBMR at the time of search (August 2014) **(Figure 4.2)**. In all, 15.5% were a 5/6 +1 NIMA match, 10.3% were a 4/6 +2 NIMA match and 2.6% were a 3/6 +3 NIMA match.

The availability of an IFM is significantly lower for ethnicity minorities compared to EC patients due to the population specific frequencies of HLA alleles and haplotypes and the predominantly EC donor population (Barker, *et al* 2010b, Gragert, *et al* 2014). It seemed plausible that the availability of a VFM would also be lower for ethnic minorities. A VFM was available for a slightly lower proportion of OE patients (25.4%) compared to EC (31.4%) patients by searching the BBMR but this difference was not statistically significant (p= 0.34). However, EC patients had a higher median number of VFMs (2, range 1 – 45) compared to OE patients (1, range 1 – 4) from the BBMR registry.

Table 4.2 Availability of virtual full match from the BBMR CB donors.
Searches were performed of 4,707 CB donors with maternal HLA banked in the NHS-CBB and listed in the BBMR through the BMDW NIMA Match Algorithm.

	Patient ethnicity			Total (*n*= 457)
	European Caucasoid (*n*= 274)	Other ethnicity (*n*= 67)	Unknown (*n*= 116)	
Virtual full match	86 (31.4)	17 (25.4)	27 (23.3)	130 (28.4)
5/6 + 1 NIMA	51 (18.6)	6 (9.0)	14 (12.1)	71 (15.5)
4/6 + 2 NIMA	29 (10.6)	9 (13.4)	9 (7.8)	47 (10.3)
3/6 + 3 NIMA	6 (2.2)	2 (3.0)	4 (3.4)	12 (2.6)
No virtual full match	188 (68.6)	50 (74.6)	89 (76.7)	327 (71.6)

Extending the search to include the 26,735 CB donors with maternal HLA listed on BMDW increased the proportion of patients with a VFM to 48.4%, although this was not directly proportional to the increase in the size of the registry searched compared to the BBMR **(Figure 4.3).** By searching the larger and international registry, which presumably has a wider genetic diversity compared to the BBMR, a greater proportion of patients (33.5%) had a 5/6 + 1 NIMA match. The remaining patients with a VFM had a 4/6 +2 NIMA match (11.4%) or a 3/6 +3 NIMA match (3.5%). A VFM was available for a comparable proportion of EC (49.6%) and OE patients (44.8%) by searching the BMDW registry (*p*= 0.63). Searching the larger BMDW registry yielded a greater number of VFMs for patients compared to searching the BBMR. EC patients had a median number of 4 (range 1 – 83) VFMs and OE patients had a median number of 2 (range 1 – 26) VFMs from the BMDW registry.

Table 4.3 Availability of virtual full match from the BMDW CB donors.
Searches were performed of 26,735 CB donors with maternal HLA (banked in the NHS-CBB plus other banks) through the BMDW NIMA Match Algorithm.

	Patient ethnicity			Total (*n*= 457)
	European Caucasoid (*n*= 274)	Other ethnicity (*n*= 67)	Unknown (*n*= 116)	
Virtual full match	136 (49.6)	30 (44.8)	55 (47.4)	221 (48.4)
5/6 + 1 NIMA	103 (37.6)	15 (22.4)	35 (30.2)	153 (33.5)
4/6 + 2 NIMA	26 (9.5)	13 (19.4)	13 (11.2)	52 (11.4)
3/6 + 3 NIMA	7 (2.6)	2 (3.0)	7 (6.0)	16 (3.5)
No virtual full match	138 (50.4)	37 (55.5)	61 (52.6)	236 (51.6)

The HLA class (I or II) of NIMA+ HLA MMs for the VFMs identified are shown in **Table 4.4.** A total of 1,654 VFMs were identified for 221 patients by searching the BMDW registry. For 5/6 + 1 NIMA matched CB donors (*n*= 585), the NIMA+ was at *HLA-A* or *HLA-B* in 74% of cases and the majority of these were at *HLA-A* (*n*= 254, 59%). For 4/6 + 2 NIMA matched CB donors (*n*= 600), 39% were NIMA matched at class I and 61% were NIMA matched at both class I and class II loci.

Table 4.4 Class I and class II NIMA matches of BMDW virtual full match donors. A total of 1,654 virtual full matches were identified for 221 of 457 patients. Searches were performed of 26,735 CB donors with maternal HLA (banked in the NHS-CBB plus other banks) through the BMDW NIMA Match Algorithm.

Virtual full match	NIMA-match			Total
	HLA-A or *-B*	*HLA-DRB1*	Class I & II	
5/6 + 1 NIMA	433 (74)	152 (26)	N/A	585
4/6 + 2 NIMA	236 (39)	N/A	364 (61)	600
3/6 + 3 NIMA	N/A	N/A	469 (100)	469

4.5 Cord blood virtual full matches for patients without an inherited full match

4.5.1 BMDW registry searches according to patient ethnicity and disease indication

Since it was determined that VFMs were available for patients, these were included in the traditional CB donor search strategy of identifying an IFM through the BMDW Match Programs (see M&M **section 2.8.2**). Patients were first evaluated for an IFM and if none were available, VFMs (in order of preference: 5/6 + 1 NIMA, 4/6 + 2 NIMA and 3/6 + 3 NIMA) were considered. If neither an IFM or VFM were identified, the donor search was extended to include inherited 5/6 and 4/6 HLA matches.

BMDW CB donor search results are shown in **Figure 4.2.** An IFM was identified for 46.6% of the total 457 patients evaluated **(Figure 4.2A)**. A VFM was identified for an additional 14.2%, increasing the cumulative availability of an IFM or VFM to 60.8%. Of these, 6.3% had a 5/6 + 1 NIMA match, 6.1% had a 4/6 + 2 NIMA

match and 1.8% had a 3/6 + 3 NIMA match. The remaining patients did not have an IFM or a VFM, most of which had an inherited 5/6 match and 2% of patients only had an inherited 4/6 match.

Donor availability was then stratified by patient ethnicity and the HLA match grades for EC and OE patients **(Figure 4.2B-C)**, respectively. In agreement with previous reports (Gragert*, et al* 2014), OE patients had a lower availability of an IFM (37.3%) compared to EC patients (48.5%). Surprisingly however, VFMs were identified for a comparable proportion of OE (16.5%) and EC (13.8%) patients, increasing the cumulative availability of an IFM or VFM to 53.8% and 62.3%, respectively.

Figure 4.2 HLA match grade of BMDW CB donors identified for A) All patients *n*= 457 **B) European Caucasoid** *n*= 274 **and C) Other Ethnicity** *n*= 67**.** Inherited matches refer to HLA matching without considering NIMA. Patients with only a mismatched donor (4/6 or 5/6) were evaluated for a virtual full match (1 - 3 NIMA substitutions at *HLA-A, -B* and/or *–DRB1*). Searches of the 621,893 listed CB donors in the BMDW and 26,735 donors with maternal HLA were performed using the BMDW Match Programs.
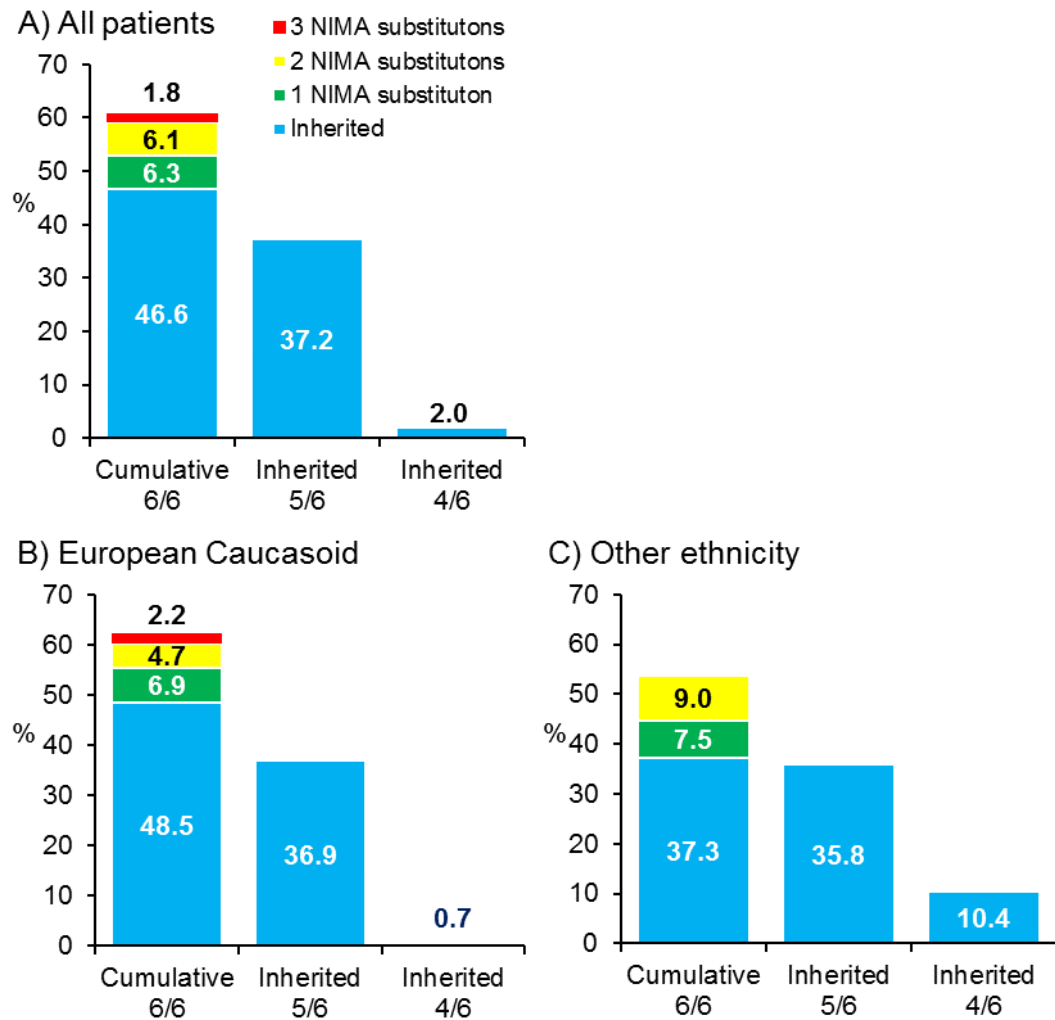
The immunological consequences of HLA MMs and their effect on clinical outcome can differ depending on the patient disease. In patients with malignant disease, the harmful effect of GvHD can partially be offset by the beneficial GvL response and HLA mismatched transplants are associated with a lower risk of relapse (Petersdorf 2008). However, in patients with non-malignant disease, there is no immunological benefit to GvHD or HLA mismatching. The availability of HLA matched CB donors was also therefore evaluated according to patient disease **(Figure 4.3)**. A higher proportion of patients with non-malignant disease had an IFM (56.8%) compared to those with malignant disease (44.2%). VFMs were identified for a similar proportion of patients with malignant disease (14.9%) compared to those treated for non-malignant indications (11.3%), increasing the cumulative availability of an IFM or VFM to 59.1% and 68.1%, respectively.

Figure 4.3 HLA match grade of BMDW CB donors identified for patients with A) malignant (*n*= 369) **and B) Non-malignant** (*n*= 88) **disease indications.** Inherited matches refer to HLA matching without considering NIMA. Patients with only a mismatched donor (4/6 or 5/6) were evaluated for a virtual full match (1 - 3 NIMA substitutions at *HLA-A, -B* and/or *–DRB1*). Searches of the 621,893 listed CB donors in the BMDW and 26,735 donors with maternal HLA were performed using the BMDW Match Programs.
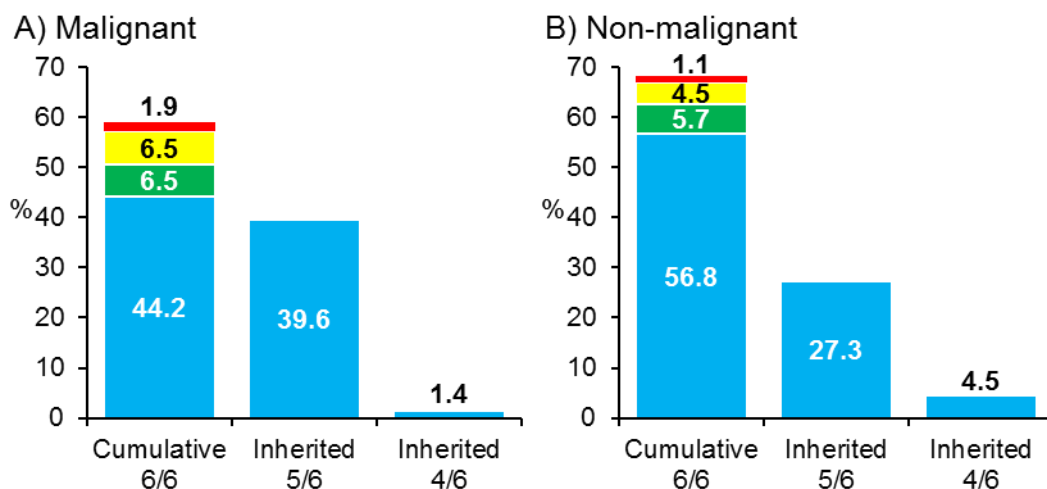
4.5.2 Inclusion of TNC dose into donor searches

Inadequate TNC dose has been linked to a higher risk of graft failure and delayed engraftment and immune reconstitution after CBT and effective balancing between HLA and TNC dose is required for optimal CB graft selection (Barker, *et al* 2010b). TNC dose was therefore incorporated into the CB donor search strategy and only donors which met the TNC dose recommendations were considered as suitable (see **M&M section 2.8.2**). The likelihood of achieving this dose reduces with increasing patient weight and a suitable graft with adequate cell dose tends to come in the form of a double graft for heavier weighted children and adults. An upper patient weight limit of 50 kilograms was therefore applied and patients above this limit were excluded from this part of the analysis.

The availability of an HLA matched CB donor that met the minimum TNC dose requirements for the 204 patients weighing 50 kg or less is shown in **Figure 4.4A.** The inclusion of TNC dose had little effect on donor availability. An IFM donor with adequate TNC dose was available for 49.5% of the 204 patients eligible for inclusion. A VFM with adequate TNC dose was identified for an additional 13.8% of patients, increasing the cumulative availability of an IFM or VFM to 63.3%. The remaining patients did not have either an IFM or VFM and 29.9% had an inherited 5/6 match, 5.9% had an inherited 4/6 match and 1.0% did not have a donor available. The HLA match grades for EC and OE patients are shown in **Figure 4.4B-C**. OE patients had a lower availability of an IFM (37.2%), compared to EC patients (55.2%). Consideration of VFMs helped a comparable proportion of patients regardless of ethnicity (EC, 13.6% versus OE, 14%). These increased the cumulative availability of an IFM or VFM from 55.2% to 68.8% for EC patients

and from 37.2% to 51.2% for OE patients. For EC patients, this equates to one additional patient benefiting from a VFM for every four patients with an IFM. OE patients saw a greater benefit due to their lower availability of an IFM, with one additional patient benefiting from a VFM for every three patients with an IFM.

Figure 4.4 HLA match grade of BMDW CB donors with adequate TNC dose identified for A) All patients *n*= 204 **B) European Caucasoid** *n*= 125 **and C) Other Ethnicity** *n*= 43**.** Inherited matches refer to HLA matching without considering NIMA. Patients with only a mismatched donor (4/6 or 5/6) were evaluated for a virtual full match (1 - 3 NIMA substitutions at *HLA-A, -B* and/or – *DRB1*). Only patients likely to be eligible for single unit cord blood transplantation and therefore weighing 50 kg or less were included. TNC dose threshold was 2.5 x 10^7 / kg for patients with malignant disease (*n*= 127) and 4.0 x 10^7 / kg for patients with non-malignant disease (*n*= 77). Searches of the 621,893 listed CB donors in the BMDW and 26,735 donors with maternal HLA were performed using the BMDW Match Programs.
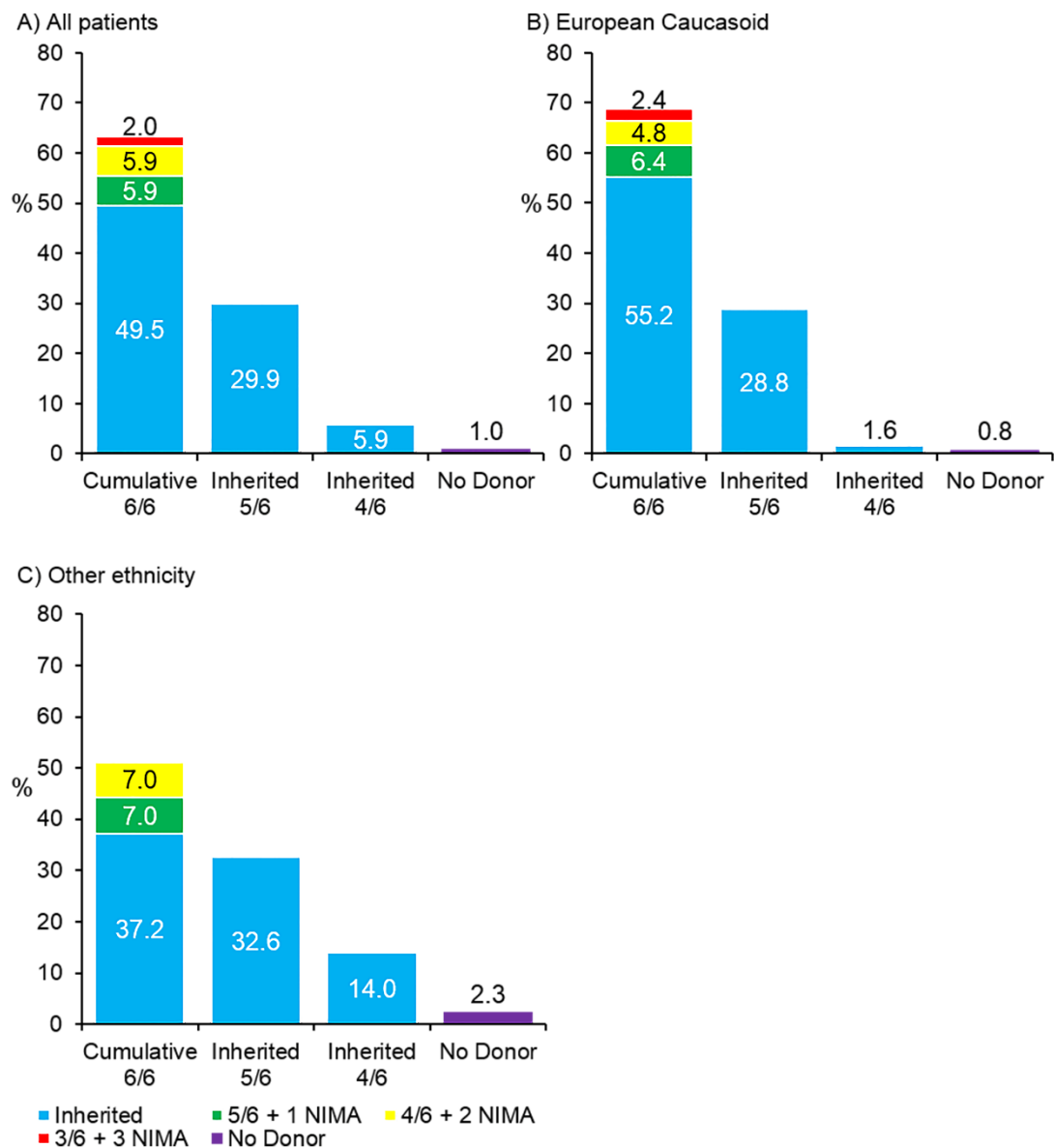
The donor search was also restricted to the BBMR CB donors to evaluate the potential benefit of performing maternal HLA typing for NHS-CBB CBUs **(Figure 4.5)**. An IFM with an adequate TNC dose was available for 20.1% of the total patients weighing 50 kg or less, which was considerably lower than when the larger international BMDW registry was searched (49.5%). Restricting the donor search to the BBMR did not however, have a detrimental effect on the availability of a VFM and instead, a higher proportion of patients had a VFM from the BBMR (19.6%) compared to BMDW (13.7%). The inclusion of VFMs doubled the cumulative availability of an IFM or VFM from the BBMR to 39.7%. Of these, 8.8% had a 5/6 + 1 NIMA match, 7.4% had a 4/6 + 2 NIMA match and 3.4% had a 3/6 + 3 NIMA match. The remaining patients did not have an IFM or VFM from the BBMR and 30.9% had an inherited 5/6 match, 26.5% had an inherited 4/6 match and 2.9% did not have a donor available.

Results were then stratified according to patient ethnicity and the HLA match grades for EC and OE patients are shown in **Figure 4.5B-C**. IFMs with adequate TNC dose were available for a quarter (24.0%) of EC patients but for OE patients, this availability was 2.5 fold lower (9.3%). VFMs were again available for a comparable number of EC (21.6%) and OE (18.7%) patients. This meant OE patients saw a greater increase in donor availability because the cumulative availability of an IFM or VFM tripled from 9.3% to 28.0%. For EC patients, VFMs doubled the cumulative availability of an IFM or VFM from 24.0% to 45.6%.

Figure 4.5 HLA match grade of BBMR CB donors with adequate TNC dose identified for A) All patients *n*= 204 **B) European Caucasoid** *n*= 125 **and C) Other Ethnicity** *n*= 43. Inherited matches refer to HLA matching without considering NIMA. Patients with only a mismatched donor (4/6 or 5/6) were evaluated for a virtual full match (1 - 3 NIMA substitutions at *HLA-A*, *-B* and/or *–DRB1*). Only patients likely to be eligible for single unit CBT and therefore weighing 50 kg or less were included. TNC dose threshold was 2.5 x 10^7 / kg for patients with malignant disease (*n*= 127) and 4.0 x 10^7 / kg for patients with non-malignant disease (*n*= 77). Searches of the 21,020 listed CB donors in the BBMR and 4,707 donors with maternal HLA were performed using the BMDW Match Programs.
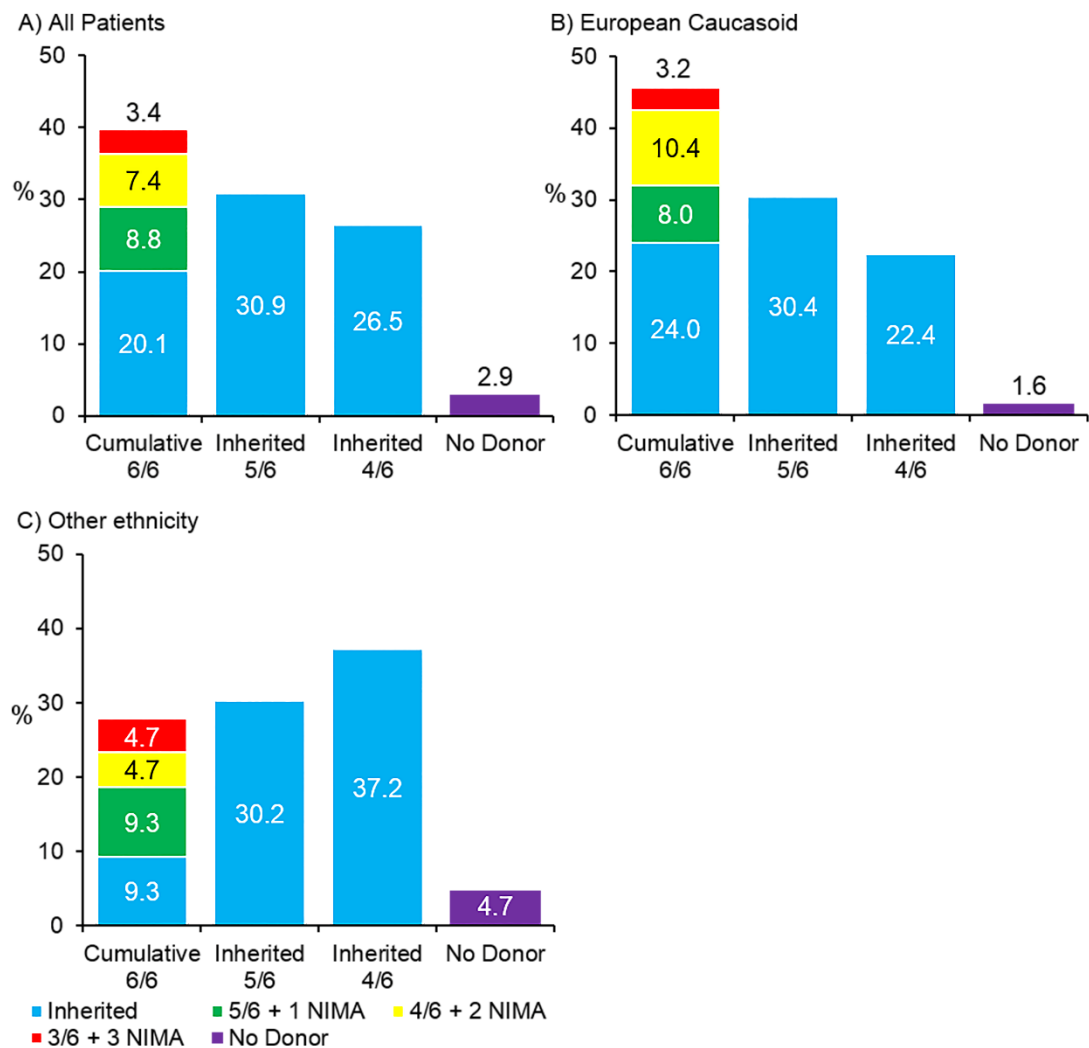
4.5.3 Virtual full matches for difficult to match patients

To understand if VFMs improved donor availability for difficult to match patients, CB donor availability for patients characterised by the presence of one or more infrequent alleles (at first field) was explored. Infrequent *HLA-A*, *-B* and *-DRB1* alleles were defined as being in the lowest cumulative frequency of 1%, 5% or 10% in the patient population (see **Appendix B**). The availability of a VFM for these "difficult to match" patients was compared to the availability of an IFM when either the BBMR or BMDW registry was searched **(Table 4.5)**. There were 239 patients with at least one HLA allele in the lowest 10% of the cumulative allele frequency and only 7 (2.9%) of these had an IFM from the BBMR. However, VFMs were identified for 30 (12.6%) patients. There were 127 patients with at least one HLA allele in the lowest cumulative 5% frequency and only 1 of these had an IFM but a VFM was identified for 10 patients. There were 54 patients with at least one HLA allele in the lowest cumulative 1% frequency and none of these had an IFM match but 3 patients had a VFM.

Table 4.5 Inherited full match (IFM) and virtual full match (VFM) CB donor availability for difficult to match patients. TNC dose was not included in donor selection.

Patient HLA with a cumulative frequency in the lowest:	BBMR			BMDW		
	IFM	VFM	*P*	IFM	VFM	*P*
1%[a]	0/54	3/54	0.079	8/54	8/54	1.000
5%[b]	1/127	10/127	**0.006**	32/127	31/127	0.884
10%[c]	7/239	30/239	**0.008**	70/239	72/239	0.841

[a] *A*34, A*36, A*66, A*69, A*74, B*46, B*48, B*54, B*60, B*62, B*67, B*73, B*81, DRB1*10*

[b] *A*23, A*25, A*31, B*41, B*42, B*45, B*47, B*53, DRB1*09*, *DRB1*12* and those in [a]

[c] *A*30, A*32, B*37, B*50, B*55, B*56, DRB1*08, DRB1*16* and those in [b]

Extending the search to the BMDW registry increased the number of patients with allele(s) in the lowest cumulative 10% frequency for whom an VFM could be identified from 7 (BBMR) to 70 (29.3%, BMDW) but the benefit of NIMA matching diminished and a VFM was identified for just 2 additional patients (n= 72, 30.1%). A similar trend was observed for patients with allele(s) in the lowest 5% or 1% cumulative frequency and IFMs and VFMs were identified for comparable numbers of patients (5%: IFM, 32; VFM, 31 and 1%: IFM, 8; VFM, 8).

4.6 Discussion

4.6.1 Virtual full matches were identified for patients from the BBMR and BMDW registries

Initial analyses demonstrated that VPs in the BBMR were able to provide VFMs for 28.4% of patients and searching the BMDW registry increased availability to 48.4%, likely due to the larger number of VPs present. The majority of VFMs identified from the BBMR were a 5/6 + 1 NIMA match (54.6%, n= 71/130) and this increased when the search was extended to BMDW (69.2%, n= 153/221). This is important because due to the current limited availability of clinical evidence, a VFM should not be preferentially selected over a donor with a higher degree of inherited match (for example, a 4/6 + 1 NIMA should not be chosen over a 5/6 with no NIMA matches). This recommendation may change if more clinical evidence becomes available.

Comparison of EC patients to those of other ethnicities revealed OE patients had a slight but non-statistically significant reduction in the availability of a VFM compared with EC patients for BBMR searches (EC, 31.4%; OE, 25.4%; p= 0.34).

This contrasts with the wide differences in the availability of an IFM described in this study and as published elsewhere (Gragert, *et al* 2014). There were three times the number of VPs than inherited phenotypes in the BBMR and a large proportion of these were from ethnic minorities (chapter 3) and this larger donor pool could explain why OE patients were not at a disadvantage with regards to VFM donor availability. However, when BMDW was searched, which has a higher number of inherited phenotypes compared to VPs, OE patients were again not at a disadvantage compared to EC patients (*p*= 0.63). Furthermore, despite the differences in size between the BMDW inherited and VPs, patients had a similar availability of an IFM (EC, 48.5%; OE, 37.3%) compared to a VFM (EC, 49.6%; OE, 44.8%). The size of the VP donor pool therefore seems not the only contributing factor to the availability of a VFM. It is possible there is an increased diversity amongst VPs, possibly because they presumably do not show patterns of LD and indeed, the previous chapter showed a large proportion of VPs were unique. Patients with less common phenotypes, at least amongst the predominantly Caucasian registries, could therefore benefit from the phenotype diversity amongst VPs.

When modelling NIMA match probability on a case-by-case basis, knowledge of the likelihood of identifying a NIMA match at each HLA locus and for individual HLA alleles will be important; particularly if maternal HLA typing is to be requested on individual CB donors as part of a further typing when searching for an appropriate donor. The majority (74%) of NIMA matches for 5/6 + 1 NIMA CB donors identified in this study were at *HLA-A* or *HLA-B*. Class I MMs are thought to be less detrimental than *HLA-DRB1* MMs and are generally the preferred MM

(Eapen, *et al* 2011b). It therefore seems feasible that if an IFM was unavailable, a class I antigen could be selected as a potential MM and this MM could readily be targeted for a NIMA+ during the donor search and selection process.

4.6.2 Virtual full matches improved donor availability for patients without an inherited full match and met the recommended minimum TNC dose recommendations

The relevance of VFMs in the traditional CB donor search strategy was explored by evaluating the availability of a VFM for patients without an IFM. When donor searches were performed using the BMDW registry, an IFM was available for 49.5% of patients. This is comparable to a recent analysis whereby an IFM from BMDW was identified for 46.4% of 2,020 Dutch patients (Van der Zanden, *et al* 2014). However, this study identified a VFM from BMDW for fewer patients without an IFM compared to 32.0% of Dutch patients (Van der Zanden, *et al* 2014). This is unlikely to be a reflection of my inclusion of TNC dose because this was shown to have little effect on the availability of a VFM for patients without an IFM (HLA only, 14.2%; TNC model, 13.8%). There could have been differences between the patient populations but if so, this did not lead to differences in the availability of an IFM between populations. There were however, significant differences in the pool of VPs searched. In this thesis, only VPs for the 26,735 BMDW CB donors with known maternal HLA were included, whereas van der Zanden and colleagues used the haplotype frequencies of the entire BMDW CB registry to estimate the VPs of 167,201 donors (Van der Zanden, *et al* 2014).

VFMs from the BBMR doubled the cumulative availability of a matched donor for EC patients and tripled the availability for OE patients. Incorporation of VFMs into the CB donor search strategy therefore provided a larger relative benefit to donor availability from BBMR searches compared to BMDW searches. This has implications for UK patients requiring CBT, whereby if an IFM was not identified from the BBMR, patients could potentially benefit from a VFM from the BBMR as opposed to initiating an international search for an IFM. In the BBMR analysis, VFMs also significantly increased donor availability for difficult to match patients with one or more infrequent HLA alleles. In contrast, IFMs and VFMs were available for a comparable proportion of patients with infrequent HLA alleles when the BMDW registry was searched. This indicates that there remains a pool of patients with rare phenotypes and for whom it is very difficult to identify a matched CB donor, despite the inclusion of VPs and warrants continuing to perform maternal HLA typing of banked CBUs to generate additional VPs available for donor searches.

For patients with malignant disease, 44.2% had an IFM and a further 14.9% had a VFM available. HLA mismatching can offer a beneficial GvL effect for patients with malignant disease and preliminary evidence suggests that, for myelogenous diseases, a stronger GvL response may be elicited after NIMA+ CBT (van Rood, *et al* 2009). The precise cellular and molecular mechanisms of this remain unclear but an anti-NIMA immunity may be upregulated after re-exposure to the NIMA MM of the recipient. If confirmed, there could be disease-specific cases where a VFM is preferred over an IFM or 5/6 match.

4.6.3 Limitations and further work

Although the availability of two (4/6 + 2 NIMA) and three (3/6 + 3 NIMA) NIMA matches were included in this analysis, their clinical relevance in CBT remains unclear. NIMA matching has previously occurred by chance and hence the majority of NIMA+ CB transplants to date have been 5/6 + 1 NIMA or 4/6 + 1 NIMA matches. However, 4/6 + 1 NIMA matching in unrelated CBT and 3/6 + 3 NIMA matching in haploidentical sibling HSCT and renal transplantation have shown positive clinical outcomes, which could extend to two and three NIMA matches in CBT (Burlingham, *et al* 1998, van Rood, *et al* 2002, Wang, *et al* 2014). Indeed, this rationale was applied when the decision was made to include these in the BMDW NIMA Match Program, which will allow future clinical evaluation of these NIMA match categories.

Matching one of two donor-recipient HLA MMs to the CB NIMA i.e. a 4/6 + 1 NIMA, to give a virtual 5/6 match, has also been shown to benefit clinical outcomes (van Rood, *et al* 2009) and are available for triple the number of patients with a VFM (Rocha, *et al* 2012, Van der Zanden, *et al* 2014). Virtual 5/6 matches were not considered in this analysis because at the time of search, they were not included in the BMDW NIMA Match Program but their availability warrants further investigation. Virtual 5/6 matches could be beneficial for patients with rare phenotypes and/or the 26.5% of the patients weighing 50 kg or less for whom the most compatible CB donor with an adequate TNC dose identified from the BBMR was an inherited 4/6 match.

CB donor searches were based on the recommended HLA matching criteria (*HLA-A* and *–B* at antigenic or intermediate resolution and *–DRB1* at high resolution) at the time of this study. However, recent evidence in favour of matching at *HLA-C* and at allele level for class I and class II HLA in CBT (Eapen*, et al* 2014) has resulted in a change in UK donor selection recommendations (Hough*, et al* 2016). Allele level matching at *HLA-A*, *-B*, *-C* and *-DRB1* was not considered in these analyses owing to the lack of maternal and CB allelic HLA typing and the absence of allelic matching in the BMDW Match algorithm (this has since been implemented). Although improved donor-recipient histocompatibility may reduce the clinical benefit to NIMA matching, the inclusion of more stringent HLA matching criteria may further reduce the availability of an optimally HLA matched CB donor and it is feasible that NIMA matching may still prove advantageous when selecting preferential HLA MMs.

Previous analyses have indicated wide differences in the probability of finding an IFM for patients of different ethnic backgrounds (Gragert, *et al* 2014). However, Non-European Caucasoid, Black, South and East Asian and those of mixed ethnicity were grouped together in this study due to the small patient cohort. Further investigations into donor availability of VFMs should therefore exploit a larger patient cohort to permit further stratification according to individual patient ethnic groups.

In this study, only previous recipients of an NHS-CBB were evaluated and importantly will not include patients that may not have proceeded to transplant due to the unavailability of a suitable donor. Match rates in this study could

therefore be overestimated, although the cohort did include a clinically heterogeneous group of patients with a broad range of HLA phenotypes.

4.6.4 Conclusions

VFMs were available for patients without an IFM and thus when incorporated into the inherited match donor search strategy, VFMs, with adequate TNC dose, were able to extend the availability of a suitable donor for patients requiring a transplant and in particular, for patients of ethnicities other than EC. This provides evidence for the existence of VPs as true phenotypes in the patient population that were not yet present in the BBMR or BMDW registries. NIMA matching could therefore be considered prospectively during the donor search and selection process. Further work should focus on a larger patient cohort to allow stratification according to ethnicity and include *HLA-C* and allele level resolution in matching probabilities. However, there is also a need for clinical evidence supporting the inclusion of NIMA matching at *HLA-C* and in the context of donor-recipient allelic resolution matching at *HLA-A*, *-B*, *-C* and *-DRB1*.

Chapter 5 Retrospective analysis of NIMA matching at *HLA-A*, *-B*, *-C* and –*DRB1* and recipient outcomes after single cord blood transplantation

Key points:

- Development of a database containing recipient, donor and maternal demographics, HLA and recipient clinical outcomes
- Inclusion of *HLA-C* identified additional HLA MMs
- 16 of 168 transplants were NIMA matched
- The study was underpowered to detect a difference in overall survival, TRM, relapse, neutrophil recovery and acute and cGvHD
- A large international collaboration is needed to evaluate NIMA matching at allelic resolution for *HLA-A/-B/-C/-DRB1* on recipient outcomes

5.1 Introduction

Numerous studies on the regulation of responses towards NIMA *in vitro* and on the influence of NIMA on clinical outcomes in the related HSCT and renal transplantation setting have been published (Burlingham*, et al* 1998, Mold*, et al* 2008, van Rood*, et al* 2002, Wang*, et al* 2014). However, there have only been two studies regarding the influence of NIMA matching on clinical outcome after unrelated CBT (Rocha, *et al* 2012, van Rood, *et al* 2009), partly due to unavailability of maternal HLA typing for transplanted CBUs. HLA and NIMA matching for these studies considered *HLA-A* and *–B* antigens and *–DRB1* alleles and not the recently introduced class I allele matching that also considers *HLA-C* (Hough*, et al* 2016). The probability of NIMA matching by chance was associated with high frequency HLA alleles (Rocha, *et al* 2012, van Rood, *et al* 2009). It is possible that the beneficial effect of NIMA matching in CBT could be a consequence of improved degree of high resolution HLA matching at *HLA-A/-B/-C/-DRB1* in the NIMA matched group; that was not identified through further HLA typing and therefore not adjusted for in statistical analyses (Brady, *et al* 2015).

It is also possible that the beneficial effect of NIMA matching may be diminished in the context of improved HLA matching in CBT, where non-HLA factors are likely to be limiting an improvement in OS. The less stringent HLA matching requirements in CBT have historically improved donor availability for ethnic minorities without an MUD (Barker, *et al* 2010a, Gragert, *et al* 2014) but the change in HLA matching for CBT, to reflect that of adult donor sources (Hough*, et al* 2016), could reverse this trend. If NIMA matching maintains its advantage on clinical outcome in the context of high resolution HLA matching, then it is possible

that NIMA matching could become even more valuable for recipients with a decreased degree of inherited high resolution HLA matches, as shown in the previous chapter (Powley*, et al* 2016).

The NHS-CBB has issued over 500 CBUs for transplantation, receives regular updates on the clinical outcome of these transplants, and has the ability to perform maternal HLA typing. This enables the rare opportunity to study the impact of NIMA matching on clinical outcomes after CBT.

5.2 Aims

The primary aim of this chapter was to retrospectively determine whether NIMA matching was associated with improved clinical outcomes after single CBT, in recipients issued with an NHS-CBB CBU to treat malignant and non-malignant disease **(Figure 5.1)**. It was hypothesised that HLA mismatched, NIMA+ transplants would be associated with a lower TRM and faster neutrophil recovery compared to HLA mismatched, NIMA- transplants. The original intention was to perform the analysis of NIMA matching using donor-recipient allele matching at *HLA-A*, *-B*, *-C* and *-DRB1*. However, despite attempts to increase the size of the cohort, the number of transplant pairs eligible for inclusion remained small and the analysis was therefore performed using matching based on *HLA-A*, *-B* and *-C* antigens and *-DRB1* alleles. The study remained statistically underpowered using the less stringent matching criteria but given the data collected and methodology training provided the bases for other parts of this thesis and future work, a retrospective survival analysis of NIMA matching was still performed.

To address the research aim, the first question was "what is the incidence of NIMA matching *HLA-A*, *-B*, *-C* and *–DRB1* by chance?" The majority of recipients, CBUs and mothers did not have the required level of HLA typing and where possible, allelic level HLA typing was completed by SBT and missing data was obtained from external sources. The donor-recipient HLA match grades were then determined, the CBU NIMA were identified and each HLA MM was checked for a NIMA match.

This enabled the second research question to be addressed, "what are the probabilities of OS (primary endpoint) and TRM, relapse, neutrophil recovery and acute and cGvHD (secondary endpoints) after HLA matched, NIMA+ and NIMA- CBT?" The influence of NIMA matching on these endpoints was considered after adjustment for other statistically associated variables in a multivariate analysis. Clinical outcomes data were provided by Eurocord and required data coding and calculation of the endpoints with censoring and inclusion of competing events.

Figure 5.1 Chapter 5 aim and questions proposed to address this aim

Aim. Retrospectively determine whether NIMA matching at *HLA-A*, *-B*, *-C* and *–DRB1* was associated with improved clinical outcomes after single CBT

Q1. What is the incidence of NIMA matching by chance?

- NIMA matching at HLA-A/B/C/DRB1
- Allelic level match grades of NIMA+ and NIMA- CBT

Q2. What were the probabilities of the primary and secondary endpoints after HLA matched, NIMA+ and NIMA- CBT?

- Primary endpoint: OS
- Secondary endpoints: neutrophil recovery, acute and cGvHD, relapse, TRM

Abbreviations: CBT, cord blood transplantation; GvHD, graft-versus-host-disease; OS, overall survival; TRM, treatment-related mortality.

5.3 HLA typing and determination of donor-recipient matching

5.3.1 HLA typing of recipients, CBUs and mothers

A total of 244 recipients received a single graft for first transplantation and were eligible for inclusion, as described in **M&M section 2.4.2**. There were 39 recipients with missing clinical outcome data for all endpoints, either due to a loss of follow-up, the absence of recipient consent for research or the absence of transplant centre consent for providing outcome data to the NHS-CBB. The incidence of NIMA matching and its effect on clinical outcomes after CBT could therefore be potentially be evaluated for 205 recipients.

Allelic resolution HLA types across four loci (i.e. *HLA-A/-B/-C/-DRB1*) were only available for 68 (33%) of the 205 recipients. An additional 16 recipient allelic resolution HLA types were identified from hard copies of recipient HLA typing reports from other H&I laboratories. Eurocord were able to provide an additional 16 recipient allelic resolution HLA types. It was not possible to contact all transplant centres ourselves due to the international agreement that all queries must be mediated through Eurocord and requests should not be sent by individual laboratories or registries. We did, however, contact UK H&I laboratories requesting additional data or pre-transplant samples. Eighteen pre-transplant DNA samples were requested and received for further HLA typing, as detailed in **M&M section 2.6.2**. Ten samples passed SBT and 8 samples failed due to poor quality DNA. Allelic resolution HLA types for a further 34 recipients were received from the UK H&I laboratories.

As a result of contacting H&I laboratories in England, contacting Eurocord and checking the hard copies of recipient files, the number of recipients with allelic resolution HLA typing data available for this study increased from 68 to 144. Of the 144 transplants, allelic resolution HLA typing at *HLA-A*, *-B*, *-C* and *-DRB1* was available for only 34 of the corresponding CBUs. PCR-SBT was completed for a further 59 CBUs. This brought the total donors and recipients with allelic resolution HLA typing at *HLA-A*, *-B*, *-C* and *-DRB1* to 93. DNA or tissue was either unavailable or of poor quality for the remaining 51 CBUs and further typing was not possible.

In an attempt to increase the size of the cohort available for the retrospective survival analyses, the matching criteria was reduced to low resolution for *HLA-A*, *-B*, and *-C* and allelic resolution for *HLA-DRB1*. All of the 205 recipients were typed to this degree but not all of the paired CBUs had *HLA-C* typing data available. *HLA-C* PCR-SBT for CBUs was therefore completed where required and *HLA-A*, *-B*, *-C* and/or *-DRB1* PCR-SBT for maternal samples was also completed. This resulted in a final cohort size of 198 recipients, CBUs and mothers HLA typed to a minimum of first field at class I and second field at *HLA-DRB1*.

Given that DNA was not available for all recipients without allelic level HLA types, the feasibility of imputing first field and intermediate resolution HLA types to allelic level using HLAMatchmaker was explored (see **M&M section 2.6.3**). To evaluate the accuracy of HLAMatchmaker, CBUs with laboratory-defined allelic HLA types were checked against the predicted alleles when allelic HLA types were collapsed back to first field. All CBUs with allelic level defined HLA at one or more HLA loci were included, regardless of study inclusion eligibility, to increase

the sample size available for concordance checking. There were 458 *HLA-A* alleles evaluated and 78.6% of computational predictions were concordant with previous HLA typing, 80.9% were concordant for the 491 *HLA-B* alleles evaluated but only 36.9% were concordant for the 452 *HLA-C* alleles evaluated **(Table 5.1)**. Imputation concordance with previous allelic results was possibly lower than expected due to recent advances in HLA typing technology since the development of HLAMatchmaker. HLAMatchmaker does not consider the allelic string (intermediate HLA typing) when predicting the HLA allele. Prediction of HLA alleles also relies on ethnicity data, which is often self-reported and not always genetically descriptive. It was therefore decided that concordance was not strong enough to use imputed allelic level HLA types from HLAMatchmaker for this analysis.

Table 5.1 Concordance of *in silico* predicted class I alleles compared to HLA tying results. *In silico* prediction was performed using HLAMatchmaker.

| Locus | Concordance | | Total |
	Concordant	Discordant	
HLA-A	360 (78.6)	98 (21.4)	458
HLA-B	397 (80.9)	94 (19.1)	491
HLA-C	167 (36.9)	285 (63.1)	452
Total	924 (66.0)	477 (34.0)	1,401

5.3.2   *HLA-A, -B, -C* and *–DRB1* donor-recipient and NIMA matching

There were 93 single graft transplants with donor and recipient allelic level four loci HLA types (*HLA-A, -B, -C* and *–DRB1*). Four loci allelic level HLA match grades (graded out of 8) were compared to historic lower level match grades (graded

out of 6 based on antigenic *HLA-A* and *−B* and allelic level *−DRB1*) to determine if further HLA MMs were revealed from the additional resolution of HLA typing **(Table 5.2)**. There were 22 transplants that were 6/6 HLA matched and 68% (*n*= 15) were also matched to 8/8 at allelic level. The remaining 32% (*n*= 7) had a single allelic HLA mismatch. HLA mismatched transplants based on historic criteria (5/6 or lower) corresponded to a lower four loci allelic match, with 20% (*n*= 19) of the total 93 transplants matched at 4/8 or lower.

Table 5.2 Allele-resolution (*HLA-A*, *-B*, *-C*, *-DRB1*) donor-recipient HLA matching compared to historic matching (*HLA-A* and *−B* at antigen level and *−DRB1* at allele-level). Class I HLA allele MMs that not identified by lower-resolution matching are shown in **red**. Percentages are shown in parenthesis.

HLA-A, *-B* (antigen) and *−DRB1* (allele) match	*HLA-A*, *-B*, *-C* and *-DRB1* (allele) match							Total
	8/8	7/8	6/8	5/8	4/8	3/8	2/8	
6/6	15 (68)	7 (32)	0	0	0	0	0	22
5/6	-	9 (23)	14 (35)	13 (33)	3 (8)	0 (0)	1 (3)	40
4/6	-	-	4 (13)	14 (47)	8 (27)	4 (13)	1 (3)	30
3/6	-	-	-	0 (0)	1 (50)	1 (50)	0	2
Total	15 (16)	16 (17)	18 (19)	27 (29)	12 (13)	5 (5)	2 (2)	93

The 77 transplants HLA mismatched at allelic level (7/8 or lower) were evaluated for a NIMA match and 10 were identified to have an NIMA+ HLA MM. NIMA+ transplants did not appear to have a higher degree of allelic level donor-recipient HLA matching (7/8 = 1, 6/8 = 3, 5/8 = 2, 4/8 = 3, 3/8 = 0, 2/8 = 1) compared to NIMA- transplants but unfortunately the small numbers did not permit statistical testing of this observation.

The small number of single graft transplants with allelic level HLA types for recipients, CBUs and mothers meant further analysis was restricted to matching *HLA-A*, *-B* and *–C* antigens and *–DRB1* alleles. The final study cohort contained 168 recipients (and the corresponding CBUs and mothers) that met this criterion. Comparison of the historic HLA match grades to the revised HLA match grades with *HLA-C* antigen matching included is shown in **Table 5.3**. Of the 6/6 HLA matched transplants, 84% were also matched at *HLA-C* to give an 8/8 HLA match (*n*= 42, *HLA-A*, *-B* and *-C* antigen and *-DRB1* allele) and the remainder were 7/8 HLA matched (*n*= 8). Only 34% of 5/6 (*n*= 24), 20% of 4/6 (*n*= 24) and none of the 3/6 HLA matched transplants were matched as 7/8, 6/8 or 5/8, respectively.

Table 5.3 *HLA-A*, *-B* and *–C* antigen and *-DRB1* allele donor-recipient HLA matching compared to historic matching without *HLA-C*. *HLA-C* antigen MMs that were not identified by lower-resolution matching are shown in **red**. Percentages are shown in parenthesis. CBT *n*= 168.

HLA-A, -B (antigen) and –DRB1 (allele) match	HLA-A, -B, -C (antigen) and -DRB1 (allele) match					Total
	8/8	7/8	6/8	5/8	4/8	
6/6	42 (84)	8 (16)	0	-	-	50
5/6	-	24 (34)	36 (51)	10 (14)	-	70
4/6	-	-	9 (20)	26 (59)	9 (20)	44
3/6	-	-	-	0	4 (100)	4
Total	42 (25)	32 (19)	45 (27)	36 (21)	13 (8)	168

HLA MMs at *HLA-A*, *-B*, *-C* antigens and *–DRB1* alleles were next evaluated for a NIMA match. Of the 126 HLA mismatched transplants, 16 (12.7%) were NIMA+ **(Table 5.4)**. The majority of the NIMA+ transplants were 6/8 (*n*= 7) or 5/8 HLA matched (*n*= 6), which is not surprising given that a higher number of HLA MMs

increases the number of potential NIMA matches. In this small cohort, NIMA+ transplants were not significantly associated with better HLA matching at *HLA-A*, *-B*, *-C* (antigen) and *-DRB1* (allele) compared to NIMA- transplants (p= 0.767). There were 19 NIMA matched antigens/alleles in the 16 NIMA+ cases. The majority (n = 9) of NIMA matches occurred at *HLA-C*, five occurred at *HLA-B*, three occurred at *HLA-A* and one NIMA match occurred at *HLA-DRB1*. Notably, the most frequent NIMA+ was *HLA-C*07* (n = 5), followed by *HLA-C*12* (n= 2) and *HLA-A*02* (n= 2), with the remaining NIMA matches only occurring once in the cohort.

Table 5.4 NIMA matched cases. Donor-recipient HLA MMs are shown in red and NIMA matches are shown in green. HLA match is based on HLA-A, -B and -C antigens and -DRB1 alleles. N.D, not determined; MM, mismatch.

Case	HLA match	NIMA+ MM
1	6/8	A*01
2	7/8	A*02
3	7/8	A*29
4	6/8	B*08
5	5/8	B*15
6	6/8	B*51
7	4/8	B*52 & C*12
8	5/8	C*03
9	6/8	C*07
10	5/8	B*07 & C*07
11	6/8	C*07
12	5/8	C*07
13	5/8	C*12
14	6/8	C*15
15	6/8	DRB1*13
16	5/8	A*02

5.3.3 Donor and recipient demographics

NHS-CBB CBUs were issued to both paediatric (57%) and adult recipients (43%) that were mainly treated in European (75%) and North American (15%) transplant centres between 1999 and 2014 **(Table 5.5)**. The majority of recipients were male (61%) and European Caucasoid (64%). Indications for transplant primarily comprised malignant diseases (70%), that were in first (36%) or higher (38%) remission. Non-malignant indications for transplant were mainly primary immune deficiencies (15%) such as severe combined immunodeficiency (SCID), or inborn errors of metabolism (10%) such as Hurler's syndrome in paediatric recipients. Conditioning regimens included both MAC (64%) and RIC protocols (37%). The majority of recipients received ATG before day 0 and received cyclosporine and/or a steroid based aGvHD prophylaxis alone (50%) or with mycophenolate mofetil (37%). The median post-processing TNC dose was 7.2×10^7 per kg recipient weight, 18.4% received a TNC dose less than 2.5×10^7 per kg, 40.0% received a dose between 2.5 and 5.0×10^7 per kg and 50.6% received a dose greater than 5.0×10^7 per kg.

There were some significant differences in demographics between the 8/8 HLA matched, NIMA- and NIMA+ HLA mismatched groups. Recipients of an 8/8 HLA matched CBU were more likely to be paediatric (86%) compared to NIMA- (48%) and NIMA+ HLA mismatched (37%) groups ($p > 0.0001$) and thus were also more likely to be treated for non-malignant diseases (67%) compared to compared to NIMA- (19%) and NIMA+ (12%) HLA mismatched groups ($p > 0.0001$). Recipients of an 8/8 HLA matched CBU were less likely to receive a total body irradiation (TBI)-based MAC conditioning regimen (8%) and were more likely to receive a

non-TBI based RIC regimen (45%, *p*= 0.002), without ATG (60%, *p*= 0.015), likely

due to their paediatric nature and treatment for non-malignant diseases. NIMA+

CBT recipients were more likely to receive a MAC regimen that was TBI based

(53%) compared to NIMA- HLA mismatched CBT recipients (28%, *p*= 0.002).

Table 5.5 Recipient, CBU and transplant characteristics according to donor-recipient HLA match status. HLA matching was based on *HLA-A*, *-B*, *-C* (antigen) and *–DRB1* (allele)**.**

Variable	Total	8/8	HLA mismatched		*P*
			NIMA-	NIMA+	
Number of recipients	168 (100)	42 (25)	110 (65)	16 (10)	
Region (*n*= 8 unknown)					0.559
Europe	124 (74)	33 (79)	80 (73)	11 (69)	
United States of America	25 (15)	3 (7)	19 (17)	3 (19)	
Other	19 (11)	6 (14)	11 (10)	2 (13)	
Recipient age					**<0.0001**
≤ 16	95 (57)	36 (86)	53 (48)	6 (37)	
> 16	73 (43)	6 (14)	57 (52)	10 (63)	
Recipient gender					0.684
Male	103 (61)	28 (67)	66 (60)	9 (56)	
Female	65 (39)	14 (33)	44 (40)	7 (44)	
Recipient ethnicity					0.327
European Caucasoid	107 (64)	28 (67)	70 (64)	9 (56)	
Other	33 (20)	6 (14)	21 (19)	6 (38)	
Unknown/ Not declared	28 (16)	8 (19)	19 (17)	1 (6)	
Recipient CMV serostatus (*n*= 16 unknown)					0.122
Negative	86 (57)	25 (64)	56 (57)	5 (33)	
Positive	66 (43)	14 (36)	42 (43)	10 (66)	
Recipient diagnosis					**<0.0001**
Malignant	**117 (70)**	**14 (33)**	**89 (81)**	**14 (88)**	
ALL	40 (24)	2 (5)	30 (27)	8 (50)	
AML	34 (20)	4 (10)	26 (24)	4 (25)	
MDS or MDS/MPD	28 (17)	4 (10)	23 (21)	1 (6)	
Lymphoproliferative disorder	7 (4)	1 (2)	5 (5)	1 (6)	
Plasma cell disorder	1 (1)	0 (0)	1 (1)	0 (0)	
Histiocytic disorder	7 (4)	3 (7)	4 (4)	0 (0)	
Non malignant	**51 (30)**	**28 (67)**	**21 (19)**	**2 (12)**	

Variable	Total	8/8	HLA mismatched		P
			NIMA-	NIMA+	
Bone marrow failure	9 (5)	2 (5)	6 (5)	1 (6)	
Haemoglobinopathy	1 (1)	0 (0)	1 (1)	0 (0)	
Primary immune deficiency	25 (15)	18 (43)	6 (6)	1 (6)	
Inborn error of metabolism	16 (10)	8 (19)	8 (7)	0 (0)	
Recipient disease status (*n*= 39 unknown)					0.135
1st CR	26 (36)	2 (40)	21 (36)	3 (30)	
2nd or higher CR	28 (38)	0 (0)	22 (38)	6 (60)	
Partial remission	2 (3)	0 (0)	2 (3)	0 (0)	
Relapse	11 (15)	1 (20)	10 (17)	0 (0)	
Refractory disease	6 (8)	2 (40)	3 (5)	1 (1)	
ATG before d0 (*n*= 42 unknown)					**0.015**
No	50 (40)	21 (60)	26 (32)	3 (30)	
Yes	76 (60)	14 (40)	55 (67)	7 (70)	
Conditioning regimen (*n*= 13 unknown)					**0.002**
MAC, TBI based	40 (26)	3 (8)	29 (28)	8 (53)	
MAC, Non-TBI based	58 (38)	15 (39)	41 (41)	2 (13)	
RIC, TBI based	12 (8)	3 (8)	6 (6)	3 (20)	
RIC, non-TBI based	45 (29)	17 (45)	26 (25)	2 (13)	
GvHD prophylaxis (*n*= 13 unknown)					0.852
CsA +/- steroids	77 (49.7)	19 (50)	51 (50)	6 (43)	
CsA +/- steroids + MTX	10 (6.5)	1 (3)	7 (7)	2 (14)	
CsA +/- steroids + MMF	58 (37.4)	16 (42)	37 (37)	5 (36)	
Tacrolimus +/- MTX +/- MMF	8 (5.2)	1 (3)	6 (6)	1 (7)	
Other	2 (1.3)	1 (3)	1 (1)	0 (0)	
Year of transplant					0.139
1999 – 2004	40 (23.8)	7 (17)	28 (25.5)	5 (31.3)	
2005 – 2009	75 (44.6)	21 (50)	44 (40.0)	10 (62.5)	
2009 – 2014	53 (31.5)	14 (33)	38 (34.5)	1 (6.3)	
Post-processing TNC dose per kilogram (x10^7 / kg)					**0.006**
< 2.5	31 (18.4)	5 (11.9)	23 (20.9)	3 (18.8)	
2.5 - 5.0	52 (40.0)	6 (14.2)	38 (34.5)	8 (50.0)	
> 5.0	85 (50.6)	31 (73.8)	49 (44.5)	5 (31.3)	

Abbreviations: ALL, acute lymphoblastic leukaemia; AML, acute myelogenous leukaemia; ATG, anti-thymocyte globulin; CMV, cytomegalovirus; CsA; cyclosporine A; CR, complete remission; MAC, myeloablative conditioning; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MPD, myeloproliferative disorder; MTX, methotrexate; RIC, reduced intensity conditioning; TBI, total body irradiation; TNC, total nucleated cell.

5.4 Clinical outcomes

5.4.1 Overall survival

The median follow-up time (or time-to-death) was 17 months (range 1 - 174). The probability of OS at 5 years was 76% (95% CI: 64% - 90%) for 8/8 HLA matched transplants compared to 53% (95% CI: 43%-64%) for NIMA- and 44% (95% CI: 25%-76%) for NIMA+ HLA mismatched transplants (*p*= 0.04) **(Figure 5.2)**. However, after risk-adjustment for TNC dose, recipient age, conditioning regimen, year of transplant and whether the recipient was being treated for a malignant disease, there was no statistical difference in the probability of OS at 5 years between each HLA match category (*p*= 0.39).

Figure 5.2 Overall survival at 5 years according to donor-recipient HLA match status. Kaplan-Meier estimates of overall survival were calculated for all recipients and were as follows: 8/8, *n*= 42, 76% (95% CI: 64 - 90); NIMA-, *n*= 110, 53% (95% CI: 43 - 64); NIMA+, *n*= 16, 44% (95% CI: 25 - 76) (*p*= 0.04). Deaths were due to any cause and was reported by Eurocord. HLA matching was based on *HLA-A*, *-B*, *-C* (antigen) and *–DRB1* (allele).
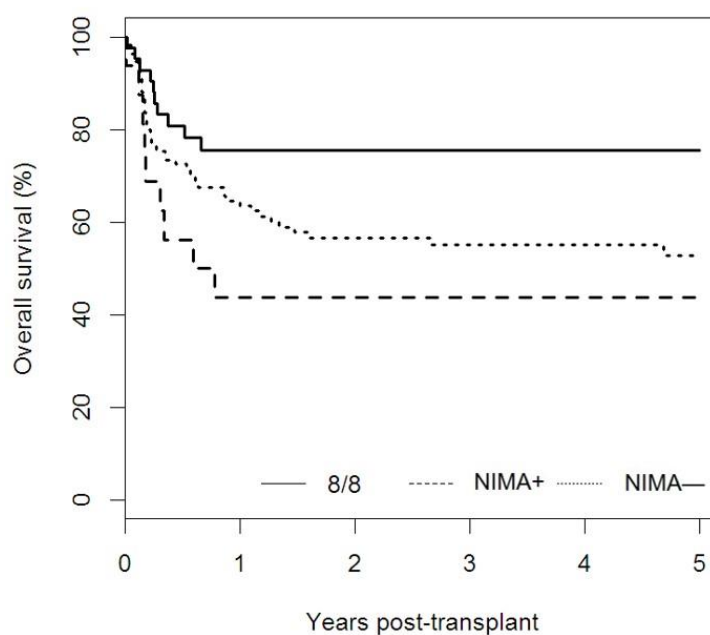
Given that an increasing number of HLA MMs has been shown to have an adverse additive effect on the probability of OS (Lee, *et al* 2007), a sub-group analysis according to the number of HLA MMs was performed. The small sample size meant 4/8 and 5/8 HLA matches and 6/8 and 7/8 HLA matches were grouped together rather than considering each match grade separately. This decision was supported by an analysis of allele level HLA matching at *HLA-A/-B/-C/-DRB1* on outcomes after single CBT for haematological malignancy that found no statistical difference in non-relapse mortality between 4/8 and 5/8 HLA matches or between 6/8 and 7/8 HLA matches (Eapen, *et al* 2014). After adding the number of MMs to the risk-adjusted model (p= 0.27) there was no difference in 5-year OS for NIMA+ transplants compared to NIMA- HLA mismatched CBT (p>0.995).

HLA-C MMs are reported to have an adverse effect on clinical outcomes after CBT (Eapen, *et al* 2014). This is the first analysis of NIMA matching on clinical outcomes after CBT to include *HLA-C* matching and it is possible the lack of statistical difference in the probability of 5-year OS between the different matching groups could be because the majority of NIMA+ cases were mismatched at *HLA-C*. A sub-group analysis according to *HLA-C* matching status was performed. After adding whether there was a MM at *HLA-C* to the risk adjusted model (p= 0.65), there was still no difference in 5-year OS (p>0.995).

5.4.2 Transplant related mortality

There was no difference in the cumulative incidence of TRM at 5 years for 8/8 HLA matched transplants (17%, 95% CI: 7%-30%) compared with NIMA- (30%, 95% CI: 21%-39%) and NIMA+ HLA mismatched transplants (38%, 95% CI:

14%-61%), (*p*= 0.20) **(Figure 5.3)**. After risk-adjustment, there was no difference in TRM for each match category (*p*= 0.71). The number of MMs (*p*= 0.15) and whether there was an *HLA-C* MM (*p*= 0.79) were added to the risk adjusted model in a sub-group of cases with at least one HLA mismatch. There was no difference in the cumulative incidence of TRM at 5 years for NIMA+ compared to NIMA- HLA mismatched transplants (*p*= 0.98) in this model.

Figure 5.3 Transplant-related mortality at 5 years according to donor-recipient HLA match status. The 5-year cumulative incidence of transplant-related mortality was as follows: 8/8, *n*= 42, 17% (95% CI: 7 - 30); NIMA-, *n*= 110, 30% (95% CI: 21 - 39); NIMA+, *n*= 16, 38% (95% CI: 14 – 61) (*p*= 0.20). The competing event for transplant-related mortality was relapse. HLA matching was based on *HLA-A, -B, -C* (antigen) and *–DRB1* (allele)**.**
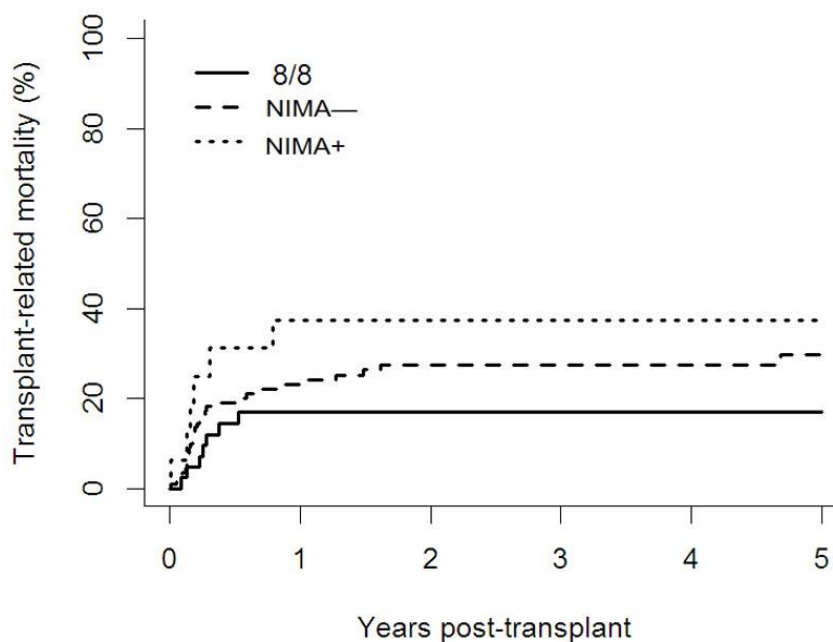
There were a total of 47 deaths attributed to TRM during the first five years post-transplant. The causes of TRM are shown in **Table 5.6** and primarily included infections (40%) and GvHD (21%).

Table 5.6 Deaths from transplant-related causes according to donor-recipient HLA match status. HLA matching was based on *HLA-A, -B, -C* (antigen) and *–DRB1* (allele). Deaths from TRM *n*= 47. Percentages are shown in parenthesis.

TRM	8/8	HLA mismatched		Total
		NIMA-	NIMA+	
Infection	8 (73)	11 (33)	3 (50)	19 (40)
Bacterial	0	3	0	3
Viral	7	3	1	8
Fungal	0	2	0	2
Parasitic	0	0	1	1
Unknown	1	3	1	5
GvHD	1 (9)	6 (18)	3 (50)	10 (21)
Multi-organ failure	1 (9)	3 (9)	0 (0)	4 (9)
Cardiac toxicity	0 (0)	3 (9)	0 (0)	3 (6)
Haemorrhage	0 (0)	2 (6)	0 (0)	2 (4)
ARDS	1 (9)	1 (3)	0 (0)	2 (4)
Rejection	0 (0)	2 (6)	0 (0)	2 (4)
PTLD EBV	0 (0)	1 (3)	0 (0)	1 (2)
VOD	0 (0)	1 (3)	0 (0)	1 (2)
Other cause(s)	0 (0)	3 (9)	0 (0)	3 (6)
Total	**11**	**33**	**6**	**47**

Abbreviations: ARDS, acute respiratory distress syndrome; GvHD, graft-versus-host disease; PTLD EBV, post-transplant lymphoproliferative disorder associated with Epstein-Barr Virus; VOD, veno-occlusive disease.

5.4.3 Relapse

There was no difference in the cumulative incidence of relapse at 5 years for 8/8 HLA matched transplants (5%, 95% CI: 1%-15%) compared to NIMA- (18%, 95% CI: 11%-26%) and NIMA+ HLA mismatched transplants (25%, 95% CI: 7%-48%) (*p*= 0.08) **(Figure 5.4)**. After risk-adjustment, there was no difference in the cumulative incidence of relapse at 5 years between each match category (*p*= 0.39). In the sub-group of cases with at least one HLA mismatch, after adding the

number of MMs to the risk-adjusted model (p= 0.71), and whether there was a MM at *HLA-C* (p= 0.71), there was no difference in relapse between NIMA+ and NIMA- HLA mismatched transplants (p= 0.47).

Figure 5.4 Relapse at 5 years according to donor-recipient HLA match status. The 5-year cumulative incidence of relapse was as follows: 8/8, n= 14, 5% (95% CI: 1 - 15); NIMA-, n= 89, 18% (95% CI: 11 – 26); NIMA+, n= 14, 25% (95% CI: 7 – 48) (p= 0.08). Relapse was diagnosed by the transplant centre as recurrence of the original disease and was reported by Eurocord. The competing event for relapse was transplant-related mortality. HLA matching was based on *HLA-A*, *-B*, *-C* (antigen) and *–DRB1* (allele).
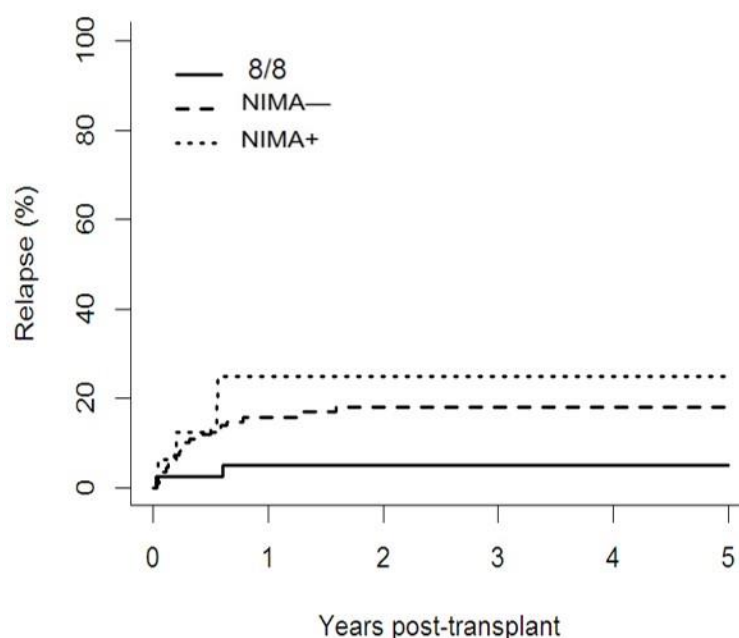
5.4.4 Myeloid engraftment

Myeloid engraftment could not be assessed in nine (5%) recipients for whom engraftment and/or graft failure data were not reported. The median time to myeloid engraftment, defined as an absolute neutrophil count greater than 0.5 × 10^9/l on three consecutive days, was 22 days in the total cohort. The median time to myeloid engraftment was 21 days in recipients of 8/8 HLA matched grafts and 22 days in recipients of NIMA- and 23 days in NIMA+ HLA mismatched grafts. The

longest reported time to myeloid engraftment was 48 days after 8/8 HLA matched CBT and 43 days after both NIMA- and NIMA+ HLA mismatched CBT. There was no difference in the cumulative incidence of myeloid engraftment for 8/8 HLA matched transplants (97%, 95% CI: 58%-100%) compared to NIMA- (84%, 95% CI: 75%-90%) and NIMA+ HLA mismatched transplants (94%, 95% CI: 32%-100%) (p= 0.12) **(Figure 5.5)**. However, after risk-adjustment, the cumulative incidence of myeloid engraftment was 70% greater for 8/8 matched transplants (sub-distribution hazard ratio (SHR) 1.69, 95% CI: 1.11-2.59) but no different for NIMA+ (SHR 1.19, 95% CI: 0.79-1.81) compared to NIMA- HLA mismatched transplants (p= 0.05). In the sub-group of cases with at least one HLA mismatch, the number of MMs (p= 0.56) and whether there was an *HLA-C* MM (p= 0.80) was added to the risk-adjusted model. There was no difference in rates of myeloid engraftment for NIMA+ compared to NIMA- HLA mismatched transplants (p= 0.54) in this model.

Figure 5.5 Myeloid engraftment according to donor-recipient HLA match status. The 48-day cumulative incidence of myeloid engraftment was as follows: 8/8, 97% (95% CI: 58 - 100); NIMA-, 84% (95% CI: 75 – 90); NIMA+, 94% (95% CI: 32 - 100) (*p*= 0.12). Myeloid engraftment was defined as an absolute neutrophil count greater than 0.5×10^9/l on three consecutive days and was reported by Eurocord. The competing event for myeloid engraftment was death. HLA matching was based on *HLA-A, -B, -C* (antigen) and *–DRB1* (allele). HLA matching was based on *HLA-A, -B, -C* (antigen) and *–DRB1* (allele).
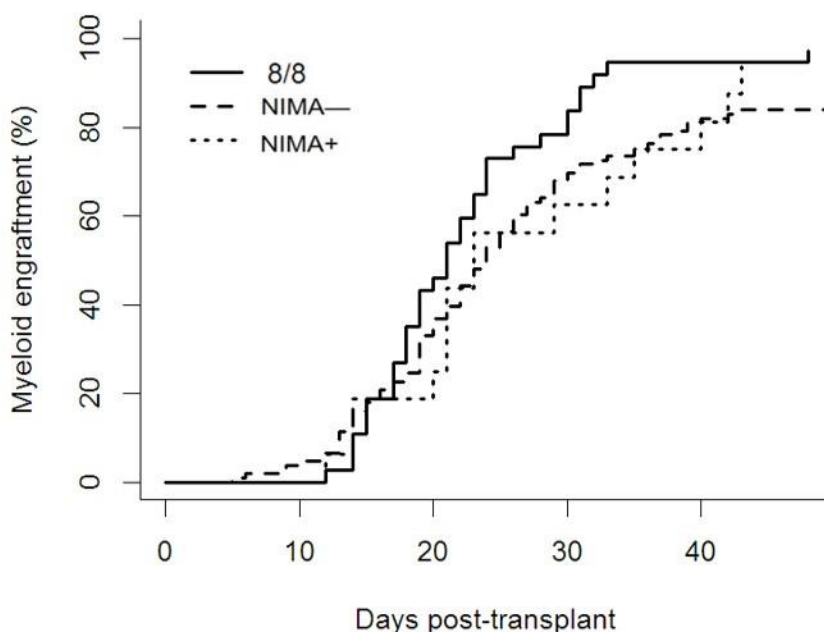
5.4.5   Acute and chronic GvHD

Nine (5%) recipients for whom engraftment and/or graft failure data were not reported were excluded from GvHD analyses. Unfortunately, due to the small numbers of NIMA+ cases, all grades of aGvHD were included together in the analysis. There was no difference in the cumulative incidence of grade I-IV aGvHD for 8/8 HLA matched transplants (54%, 95% CI: 37%-69%) compared to NIMA- (52%, 95% CI: 42%-61%) and NIMA+ HLA mismatched transplants (69%, 95% CI: 38%-87%) (p= 0.59) **(Figure 5.6)**. After risk-adjustment, there was no difference in rates of aGvHD for each match category (*p*= 0.24).

For the sub-group of cases with at least one HLA mismatch, after adding the number of MMs to the risk-adjusted model (p= 0.76) and whether there was a MM at *HLA-C* (p= 0.73), there was some difference in rates of aGvHD for NIMA+ compared to NIMA- HLA mismatched transplants, (p= 0.07). The hazard of aGvHD was 80% greater for NIMA+ (SHR 1.77, 95% CI: 0.95-3.29), compared to NIMA- HLA mismatched transplants.

Figure 5.6 Grade I-IV aGvHD according to donor-recipient HLA match status. The 100-day cumulative incidence of grade I – IV aGvHD was as follows: 8/8, 54% (95% CI: 37 - 69); NIMA-, 52% (95% CI: 42 - 61); NIMA+, 69% (95% CI: 38 - 87) (p= 0.59). AGvHD was graded by the transplant centre and was reported by Eurocord. The competing event for aGvHD was primary or secondary graft failure. HLA matching was based on *HLA-A*, *-B*, *-C* (antigen) and *–DRB1* (allele).
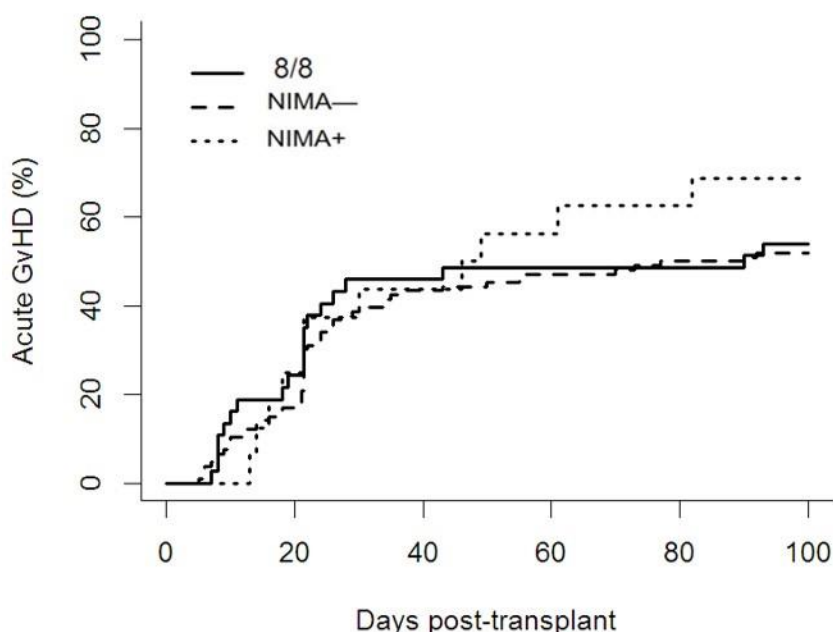
Of all recipients in the study, 43 had died or experienced graft failure within 100 days of transplant, two recipients' follow-up time was less than 100 days, one recipient was reported as experiencing cGvHD at 91 days' post-transplant and engraftment and/or graft failure data were not reported for a further eight

recipients. Rates of cGvHD were therefore assessed in 114 (69%) recipients reported as alive without graft failure at 100 days. Rates of cGvHD for 8/8 HLA matched transplants were lower (10%, 95% CI: 3%-25%) compared to NIMA- (29%, 95% CI: 19%-40%) and NIMA+ (59%, 95% CI: 7%-90%) HLA mismatched transplants (p= 0.04) **(Figure 5.7)**. After risk-adjustment, the hazard of cGvHD was 4 times greater for NIMA- transplants (SHR 4.31, 95% CI: 1.09-17.06) and 11 times greater for NIMA+ transplants (SHR 11.44, 95% CI: 1.92-68.34), compared to 8/8 HLA matched transplants (p= 0.03). For the sub-group of cases with at least one HLA mismatch, after adding the number of MMs to the risk-adjusted model (p= 0.31) and whether there was an *HLA-C* MM (p= 0.48) the hazard of cGvHD was 3 times greater for NIMA+ transplants (SHR 2.94, 95% CI: 1.04-8.29), compared to NIMA- HLA mismatched transplants (p= 0.04).

Figure 5.7 Chronic GvHD according to donor-recipient HLA match status.
The 5-year cumulative incidence of cGvHD was as follows: 8/8, 54% (95% CI: 37 - 69); NIMA-, 52% (95% CI: 42 - 61); NIMA+, 69% (95% CI: 38 - 87) (*p*= 0.59). CGvHD was only evaluated in recipients alive at day 100. The competing event for cGvHD was primary or secondary graft failure or death without cGvHD. CGvHD was graded by the transplant centre and reported by Eurocord. HLA matching was based on *HLA-A*, *-B*, *-C* (antigen) and *–DRB1* (allele)**.**
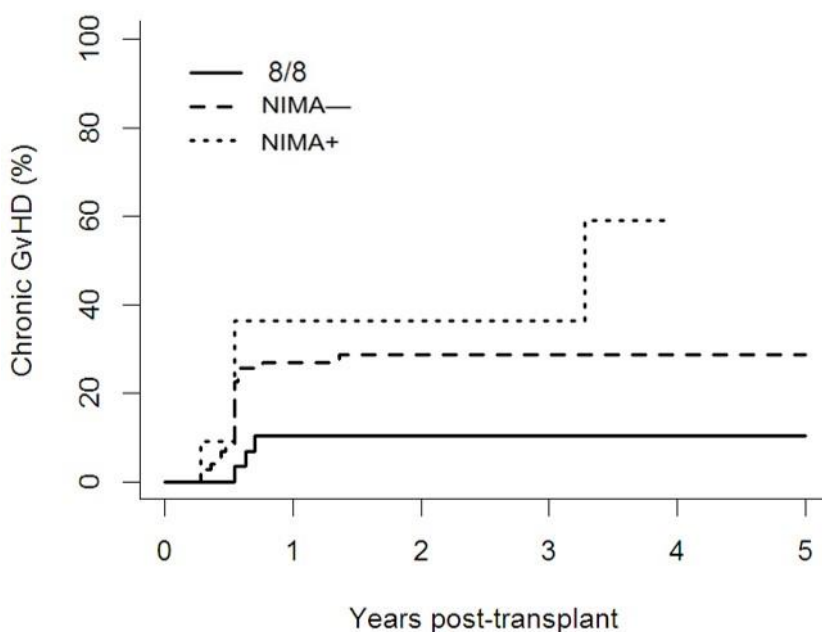
5.4.6   Statistical test of power

The absence of a statistically significant difference in the primary endpoint, OS, between NIMA+ and NIMA- HLA mismatched CBT for this cohort contradicted previously published retrospective studies (Rocha, *et al* 2012, van Rood, *et al* 2009). Two possibilities for this exist: 1) there is no true difference in OS between NIMA+ and NIMA- HLA mismatched transplants in this cohort or 2) there is a difference in OS but the statistical methodology failed to detect the difference due to a lack of power. The sample size required to detect a statistical difference was calculated using a method based on the log-rank test (Collett 2015).

OS between the NIMA+ and NIMA- groups was compared assuming an 80% power and 5% significance. A ratio of 1:7 NIMA+ to NIMA- cases was assumed based on the two published retrospective studies (Rocha, *et al* 2012, van Rood, *et al* 2009). Rocha *et al.* reported 5-year OS to be 55% after NIMA+ and 38% after NIMA- HLA mismatched CBT. The sample size required to detect a difference in OS of this magnitude is 584 (73 NIMA+, 511 NIMA- HLA mismatched cases). The study described in this thesis reports only 16 NIMA+ and 112 NIMA- HLA mismatched cases, which would only be able to detect a difference of this magnitude with 26% power, that is, there would be a 74% chance that I would fail to detect a difference if OS probabilities were 55% and 38% for NIMA+ and NIMA- HLA mismatched transplants, respectively. To maintain 80% power for this sample size would require OS of at least 73% after NIMA+ transplantation if OS after NIMA- HLA mismatched transplantation was still 38%. The study described in this thesis was underpowered to detect a significant difference in OS between NIMA+ and NIMA- HLA mismatched cases comparable to that of previous reports in the literature.

5.5 Discussion

In recipients with haematological malignancies, NIMA+ grafts have been associated with a lower TRM and improved engraftment, leading to reduced overall mortality compared with NIMA- HLA mismatched CBT (Rocha, *et al* 2012, van Rood, *et al* 2009). This analysis by van Rood and colleagues and a second analysis confirming the lower incidence of TRM after NIMA matching were performed using the historic criteria of *HLA-A* and *–B* at antigenic resolution and *–DRB1* at allelic resolution. Given the recent evidence promoting allelic level HLA

matching in CBT, the primary aim of this chapter was to retrospectively determine whether NIMA matching at *allelic level resolution for HLA-A, -B, -C and –DRB1* was associated with improved clinical outcomes after single CBT in recipients issued with an NHS-CBB CBU. The majority of recipients, donors and mothers were not HLA typed to the resolution required for this study and few recipient DNA samples were available for further testing. Unfortunately, despite best efforts to complete allelic level typing, the number of donor-recipient pairs with allelic resolution was too small (*n*= 105) to perform an analysis. Statistical evaluation of clinical outcomes was performed for HLA matching at antigen resolution at *HLA-A*, *-B* and *-C* and allele resolution at *–DRB1*.

5.5.1 HLA matching between the recipient, CBU and mother

For the subset of transplants with donor-recipient allelic level resolution HLA tying, the more stringent matching criteria identified substantial additional HLA MMs, especially for transplants that were not 6/6 HLA matched based on historical criteria. However, 68% of 6/6 HLA matched transplants were also matched at 8/8 allelic resolution level. This is higher than a previous report by Eapen *et al.*, which found that only 54% of 217 6/6 HLA matched transplants were also 8/8 HLA matched at allelic level resolution (Eapen*, et al* 2014) and is possibly a reflection of differences in the populations studied and/or the smaller cohort in this study. The NIMA+ group (*n*= 10) did not appear to have a better allelic level resolution matching compared to the NIMA- HLA mismatched group (*n*= 83) but the small cohort restricted statistical testing of this observation. However, the degree of antigenic resolution at *HLA-A*, *-B* and *-C* and allele

resolution at –*DRB1* donor-recipient HLA matching was not significantly higher in the NIMA+ group compared to the NIMA- HLA mismatched group.

This analysis considered NIMA matching at *HLA-C* and results showed that this doubled the number of chance NIMA matches, possibly because *HLA-C* showed the lowest degree of matching (66.6%) compared to the other HLA loci. The lower proportion of transplants mismatched at *HLA-DRB1* (15.0%) and the identification of only one NIMA match at *HLA–DRB1* further supports this observation. The observation that NIMA matched antigens and alleles were those present at higher frequencies in the donor population is not surprising given mathematical probabilities and is in agreement with previous reports of NIMA matching in CBT (Rocha, *et al* 2012, van Rood, *et al* 2009) and the results of chapters 3 and 4 (Powley, *et al* 2016). Taken together, this also helps clarify the concern discussed in chapter 3 that high frequency HLA antigens could be less likely to be informative for NIMA. It is possible that if there was a lower probability that high frequency antigens/alleles would be informative for NIMA this would be outweighed by the fact that the NIMA antigens/alleles were already present at a high frequency in the NHS-CBB to begin with.

5.5.2 Analyses of clinical outcomes after NIMA+ CBT

When the clinical outcomes of NIMA+ transplants were compared to those of NIMA- HLA mismatched and 8/8 HLA matched transplants, there was no significant difference in the primary endpoint, 5-year OS, in both univariate and multi-variate analyses. There was also not a significant difference in the probability of 5-year OS between 8/8 matched and HLA mismatched transplants,

despite reports to the contrary in the literature (Eapen*, et al* 2014). This is most likely to be due to the small sample size of a total 168 transplants and major differences in confounding variables (recipient age, diagnosis, conditioning regimen and TNC dose) between groups. Differences between the three populations in these confounding variables may have had a significant effect on clinical outcomes. For example, recipients of an 8/8 matched CB were significantly more likely to have received a unit with a TNC dose greater than 5 x 10^7 per kg, which is associated with a faster time to neutrophil engraftment (Barker*, et al* 2010b). Attempts were made to adjust for confounding variables through multivariate analyses but this was hampered by the small sample size.

Because NIMA matching in this cohort occurred by chance, only 16 of these were NIMA matched. Statistical analyses showed that to detect a difference in OS at 5% significance with 80% power and comparable to that previously reported in the literature (Rocha*, et al* 2012, van Rood*, et al* 2009), 584 HLA mismatched transplants would need to be analysed. At the start of this study it was reasoned that a larger sample size would be available because the NHS-CBB has issued over 500 CBUs for transplantation. However, the recent development of double CBT and thus extension of CBT to adult HSCT candidates meant that over half of the CBUs were used as part of a double transplant. Very few of these double transplants were performed using two NHS-CBB issued CBUs and therefore information on the HLA type of the second CBU and the mother was not available. Statistical analyses were restricted to the 244 single CBTs but 39 of these did not have clinical outcome data and crucially five of those missing outcome data were NIMA matched. Of the 205 with outcome data, 168 (81%) were included in the

final analyses, with the remainder excluded primarily due to a lack of recipient *HLA-C* typing but also due to the unavailability of maternal and CB DNA for further HLA typing.

Alternatively, the lack of association between NIMA matching and 5-year OS in this cohort could be due to biological reasons rather than statistical power. The small sample size meant that HLA match grades were grouped together as 1) 8/8, 2) 7/8 or 6/8 and 3) 5/8 and 4/8 when performing multivariate analyses. This grouping was based on a previous publication by Eapen *et al*., who reported comparable probabilities of OS within these groups (Eapen*, et al* 2014). However, previous NIMA analyses have shown the probability of OS for transplants with a 4/6 + 1 NIMA match graft is comparable to a 5/6 HLA match i.e. the NIMA+ HLA MM is permissible (van Rood*, et al* 2009). It is possible that any benefit of NIMA matching on 5-year OS was hidden within the HLA matching group structure in the multivariate analysis. Given that the analysis by van Rood *et al.* reported a stronger beneficial effect of NIMA matching in the 5/6 (single mismatch) group, it is possible that in this analysis the benefits of 5/6 + 1 NIMA matches were masked by the inclusion of 4/6 +1 NIMA matches. However, the univariate analysis did not show any effect of NIMA matching and that there was no significant difference in the degree of HLA matching between the NIMA+ and NIMA- HLA mismatched groups, so this seems unlikely.

Previous studies have shown a difference in clinical outcome between different HLA mismatched loci in adult donor HSCT and CBT (Flomenberg*, et al* 2004, Lee*, et al* 2007, Petersdorf 2008). The majority of NIMA matches occurred at *HLA-C* and given the previous reports of the particularly detrimental effect of *HLA-C*

MMs compared to MMs at other HLA loci (Flomenberg, *et al* 2004, Petersdorf, *et al* 2014, Woolfrey, *et al* 2011), I sought to account for this in the survival analyses. However, the presence of an *HLA-C* MM was not associated with worse clinical outcome in this cohort and risk adjustment for this variable made no difference to the models.

Although van Rood and colleagues did not establish the mechanism of improved survival after NIMA+ transplantation, the authors reasoned that the benefits were likely due to the faster neutrophil recovery and reduced TRM in the NIMA+ group (van Rood, *et al* 2009). In this analysis of NHS-CBB issued CBUs, NIMA matching was not associated with improvements in neutrophil recovery or TRM. It is possible that the use of CBUs with a high TNC dose (50.6% received a dose greater than 5.0×10^7 per kg) offset the detrimental effects of HLA mismatching and thus any potential benefit of NIMA matching. A higher TNC dose has previously been shown to improve neutrophil recovery after HLA mismatched CBT (Barker, *et al* 2010b) and because of this, it is recommended that CBUs with a higher TNC dose are selected when transplanting with a greater degree of donor-recipient HLA disparity (Hough, *et al* 2016). Indeed, unlike van Rood and colleagues, where a greater number of recipients received a low TNC dose (van Rood, *et al* 2009), Rocha *et al.* did not observe a significant effect of NIMA matching on neutrophil recovery (Rocha, *et al* 2012). The small sample size did not permit a subset analysis on the influence of NIMA matching on neutrophil recovery according to the TNC dose in the NHS-CBB cohort.

This is the first retrospective analysis to include both malignant and non-malignant disease indications when evaluating the influence of NIMA matching

on clinical outcomes after CBT. It is possible that a beneficial effect of NIMA matching was not observed in this study because there is no biological benefit to NIMA matching in transplants for non-malignant diseases. There is some evidence for a reduced relapse rate after NIMA+ CBT in recipients with malignant disease (van Rood, *et al* 2012, van Rood, *et al* 2009) and this could have been one of the reasons for the improved OS reported for NIMA matching. In non-malignant disease, any increase in aGvHD is not offset by a GvL effect and thus a reduced probability of relapse that could benefit the probability of survival.

Of some concern is the trend towards a higher risk of grade I-IV aGvHD and cGvHD after NIMA+ compared to NIMA- HLA mismatched transplants in the multivariate analysis that adjusted for the number of MMs and presence of a MM at *HLA-C*. When unrelated CBT became a clinical procedure, there was concern that maternal cells in CB graft might cause GvHD in the recipient. This has not been reported in clinical studies but recent evidence suggest maternal cells sensitised against the CB inherited paternal antigens could mediate a GvL effect if the recipient shares the CB IPA, although the authors did not report a higher incidence of aGvHD associated with the reduced incidence of relapse (van Rood, *et al* 2012). The trends towards a higher incidence of aGvHD after NIMA matching most probably reflects the inclusion of grade I aGvHD due to the small sample size, whereas previous studies have only included grades II-IV. It is also possible that due to the heterogeneous nature of the transplants analysed, there were some variables affecting the risk of acute and cGvHD that were not adjusted for in the statistical analysis.

This also contradicts the majority of reports from related haploidentical sibling HSCT, where NIMA matching was associated with a *lower* incidence of aGvHD (Ichinohe*, et al* 2004, van Rood*, et al* 2002) but is consistent with reports of a higher incidence of cGvHD in NIMA+ haploidentical HSCT (Kanda*, et al* 2009). However, the results of related haploidentical HSCT are not directly comparable to CBT due to differences in HSC source and the use of a related donor. In related haploidentical transplantation, the donor shares a complete HLA haplotype with the recipient and so HLA are matched at allelic resolution (unlike this study where HLA were matched at antigenic resolution for class I HLA); other HLA loci and non-HLA genes not included during matching are also matched. In NIMA+ haploidentical HSCT, each HLA MM is also NIMA match, whereas this study included grafts with multiple HLA MMs where only one or two were NIMA matched. However, there have also been some reports of sensitisation against NIMA and the development of severe aGvHD and a frequent occurrence of extensive cGvHD after NIMA+ haploidentical sibling HSCT (Kanda*, et al* 2009). Predicting whether sensitization or tolerance towards NIMA will occur remains a significant research question.

It is possible the use of ATG and other *in vivo* T cell depleting agents also depleted Tregs specific for NIMA. In murine studies NIMA specific Tregs have been shown to be essential for tolerance towards NIMA (Andrassy*, et al* 2003, Matsuoka*, et al* 2006, Molitor-Dart*, et al* 2007, Zhang and Miller 1993) and in the clinical setting of haploidentical sibling HSCT, the NIMA effect is especially apparent in recipients who were not taking cyclosporine (Burlingham*, et al* 1998). However, in this analysis there was no difference in the use of ATG between NIMA+ and NIMA-

HLA mismatched transplants and the trend towards a higher incidence of aGvHD after NIMA matching does not support this hypothesis.

5.5.3 Conclusions

In conclusion, this study agreed with previous reports in that 9 (8%) of 116 HLA-mismatched transplants were NIMA matched at *HLA-A*, *-B* or *-DRB1* by chance and also identified further chance NIMA matching at *HLA-C* in 9 cases (2 of which were also NIMA matched at *HLA-B*). The heterogeneous cohort of small sample size and low incidence of chance NIMA matching meant that the study was considerably underpowered to detect any differences in the clinical outcomes of NIMA+ transplants compared to NIMA- HLA mismatched transplants. A large, multicentre collaboration, most probably between Eurocord and CIBMTR (as done previously) that includes the more recent HLA matching criteria of allelic level resolution for both class I and class II is needed to explore the concept of NIMA matching in this setting. Furthermore, it is yet to be explored whether NIMA matching has an effect in the setting of double CBT. This is of particular interest given that the majority of NHS-CBB are now being issued as part of a double CBT.

Chapter 6 Development of a sensitive q-PCR assay for the detection and quantification of maternal microchimaerism in cord blood

Key points

- Optimisation of a q-PCR assay targeting HLA polymorphism, with a sensitivity of 0.01% and in accordance with MIQE publishing guidelines
- Microchimaerism was detected in 27% of the total 96 CB DNA tested and was more likely to occur in CB samples of babies born at earlier gestational time points.
- Microchimaerism was significantly associated with bi-directional histocompatibility between the mother and foetus at *HLA-A*.

6.1 Introduction

The bidirectional exchange of cells at the maternal-foetal interface during pregnancy is a normal phenomenon that increases steadily throughout gestation, resulting in the presence of maternal cells in healthy, immunocompetent offspring (MMc) that can persist well into adult life (Drabbels, *et al* 2011, Loubiere, *et al* 2006). The presence and degree of MMc has been associated with tolerance versus sensitisation towards NIMA in the experimental and haploidentical HSCT clinical setting (Ichinohe, *et al* 2004, Matsuoka, *et al* 2006, Molitor-Dart, *et al* 2007) but little is known about the clinical relevance of MMc in unrelated CBT. Since MMc does not seem to occur in all pregnancies, there are likely to be genetic, cellular and/or molecular determinants that control cell migration across the placenta and/or the survival of microchimaeric cells in the host (Mold, *et al* 2008).

When CB was first used as a graft source in HSCT, there was a concern that the infusion of maternal cells with the CB graft into the recipient could mediate aGvHD (van Rood, *et al* 2012). The mother of the CB could have been sensitised to foetal IPAs during pregnancy and because donors and recipients are HLA matched in CBT, these same HLA antigens are likely to also be expressed in the recipient. Secondary exposure of maternal cells to these IPAs when infused into the recipient could therefore have the potential to elicit a potent immune response. Clinical outcomes however, demonstrated that CB was actually associated with a lower incidence and severity of aGvHD compared to adult graft sources and these concerns were largely forgotten without any attempt to correlate the presence MMc of in the graft with clinical outcomes after

transplantation (Eapen, *et al* 2011c, Eapen, *et al* 2010, Eapen, *et al* 2007, Rocha, *et al* 2009). More recently, the observation that in haploidentical HSCT, maternal grafts were associated with lower relapse rates compared to paternal grafts (Stern, *et al* 2008), led van Rood to hypothesise that MMc present in CB grafts could mediate a GvL effect and also possibly, a GvHD response in recipients (van Rood, *et al* 2012). Acute myeloid leukaemia patients who shared one or more HLA-A, -B, or -DRB1 antigens with their CB donor's IPAs had a decreased incidence of relapse compared to patients that received a non-shared IPA transplant (van Rood, *et al* 2012). Importantly, the anti-leukemic effect of shared IPA transplants was not associated with an increased incidence of aGvHD. This evidence that maternal cells mediate an anti-leukemic effect in CBT was however, indirect and it was assumed that CB grafts were positive for MMc. The cells involved and their mechanism(s) of action also remain unknown.

The availability of sensitive methods to detect microchimaerism is critical to investigating its clinical relevance. Historical methods of detection include short tandem repeats, fluorescence in situ hybridisation and SSP but the limited sensitivity (1 – 5%) of these methods may have underestimated the presence of chimaerism and more recently, sensitive molecular methods such as q-PCR that target HLA or insertion deletion polymorphisms have been developed (Alizadeh, *et al* 2002, Berry, *et al* 2004, Eikmans, *et al* 2014, Lambert, *et al* 2004, Scaradavou, *et al* 1996). A study quantifying MMc, using sensitive methods, in clinical grade CBUs that have been transplanted would therefore be a valuable tool and would permit, for the first time, a direct analysis of the influence of MMc in the CB graft on recipient clinical outcomes after transplantation in future work.

6.2 Aims

The aim of this chapter was to develop a sensitive q-PCR assay for the detection and quantification of MMc in CB, to determine the levels of MMc in transplanted CBUs and to investigate variables associated with its presence and/or absence **(Figure 6.1)**. This required the optimisation and validation of a q-PCR assay that targeted HLA polymorphisms according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines as closely as possible (Bustin, *et al* 2009).

The optimised q-PCR assay was used to answer the first question: what proportion of transplanted CBUs tested positive for MMc? Statistical analyses were then performed to answer the second question: what variables are associated with the presence of MMc?

Figure 6.1 Aims of this research chapter and the questions proposed to address this aim

Aim: to develop a sensitive q-PCR assay for the detection and quantification of MMc in CB, to determine the levels of MMc in transplanted CBUs and to investigate variables associated with its presence and/or absence.

Optimise and validate a sensitive q-PCR assay targeting NIMA HLA for the quantification of MMc in CB DNA

What proportion of transplanted CBUs tested positive for MMc?

Which variables are associated with the presence of MMc?

- Reaction conditions
- Specificity
- Assay performance: efficiency, sensitivity, precision

- Maternal-foetal histocompatibility
- Maternal demographics

6.3 Optimisation of HLA-specific q-PCR assays and characterisation of assay performance

q-PCR assays targeting NIMA HLA were optimised and used to test for and quantify MMc in the CB. Theoretical aspects of the assay are described in **M&M section 2.7** but in brief, standard curves were used to quantify the amount of target in unknown samples and this was normalised against the total DNA of the sample, quantified by an endogenous control, HCK (also quantified in each unknown sample by a standard curve). Initial q-PCR reactions of artificial spiking experiments were set up to test the protocol, optimise the conditions and characterise the assay performance by following the MIQE guidelines as closely as possible (Bustin, *et al* 2009).

6.3.1 HLA targets, primer sequences and specificity

Eleven HLA targets: *HLA-A*01, -A*02, -A*11, -A*24, -A*24/A*25, -A*30, -A*33, -B*08, -B*27, -B*35* and *-DRB1*03*, were chosen based on the previously reported sensitivities of the assays (Drabbels, *et al* 2011, Eikmans, *et al* 2014). Forward and reverse primer sequences for each of the HLA targets were available from Michael Eikmans and colleagues and are given in **Table 6.1** (Drabbels, *et al* 2011, Eikmans, *et al* 2014).

Primer specificity was tested by setting up q-PCR reactions using DNA samples positive for the target of interest. Male platelet donors were chosen because of the availability of excess blood for multiple DNA extractions. A dissociation curve was run post-amplification, which provides information on the amplicon melting temperature (T_m). A single narrow peak was present for all expected positive

reactions. Representative examples for each target of interest are shown in **Figure 6.2.** In addition, non-template (water) controls were also run for each primer pair and all showed a negative reaction pattern in the dissociation curve. No further optimisation of the thermal cycling parameters was necessary.

Table 6.1 q-PCR HLA-specific primer sequences and amplicon length
(Drabbels, *et al* 2011, Eikmans, *et al* 2014).

Target		Primer sequence (3' – 5')	Amplicon length (bp)
HLA-A			
*A*01*	F	GGTTCTCACACCATCCAGATA	205
	R	CTCTCTGCTGCTCCGCCG	
*A*02*	F	TCCTCGTCCCCAGGCTCT	205
	R	CCCGTCCCAATACTCCGGA	
*A*11*	F	CTTCCTCCGCGGGTACCG	165
	R	CCCTCCAGGTAGGCTCTCT	
*A*24*	F	AAGGATTACATCGCCCTGAAA	125
	R	CCCTCCAGGTAGGCTCTCT	
*A*24/A*25*	F	CCACTCCATGAGGTATTTCTC	200
	R	TTCACTTTCCCTGTCTCCTC	
*A*30*	F	GTGTCCCGGCCCGGCAGT	155
	R	GGTCCCAATACTCAGGCCT	
*A*33*	F	CCACTCCATGAGGTATTTCAC	200
	R	GCCTTCACATTCCGTGTGTT	
HLA-B			
*B*08*	F	CGTGTGGCGGAGCAGGAC	105
	R	CCGCGCGCTCCAGCGTG	
*B*27*	F	CGCCGCGAGTCCGAGAGA	135
	R	GGAGCAGGGTCCGCAGGTC	
*B*35*	F	GACCGGAACACACAGATCTT	85
	R	GCTCTGGTTGTAGTAGCCGC	
HLA-DRB1			
*DRB1*03*	F	TACTTCCATAACCAGGAGGAGA	150
	R	TGCAGTAGTTGTCCACCCG	

Figure 6.2 HLA-specific q-PCR amplicon dissociation curves. (A) *A*01*, **(B)** *A*02*, **(C)** *A*11*, **(D)** *A*24*, **(E)** *A24/A*25*, **(F)** *A*30*, **(G)** *A*33*, **(H)** *B*08*, **(I)** *B*27*, **(J)** *B*35* and **(K)** *DRB1*03*. Q-PCR reactions were set up using male platelet donor DNA positive for the target of interest. Amplicons were continuously heated from $55^0C - 95^0C$ and temperature (^0C) was plotted against the derivative Reporter (-Rn') to determine the amplicon melting temperature (T_m).

(A) *A*01*

(B) *A*02*

(C) *A*11*

(D) *A*24*

(E) *A*24/A*25*

(F) *A*30*

(G) *A*33*

(H) *B*08*

(I) *B*27*

(J) *B*35*

(K) *DRB1*03*
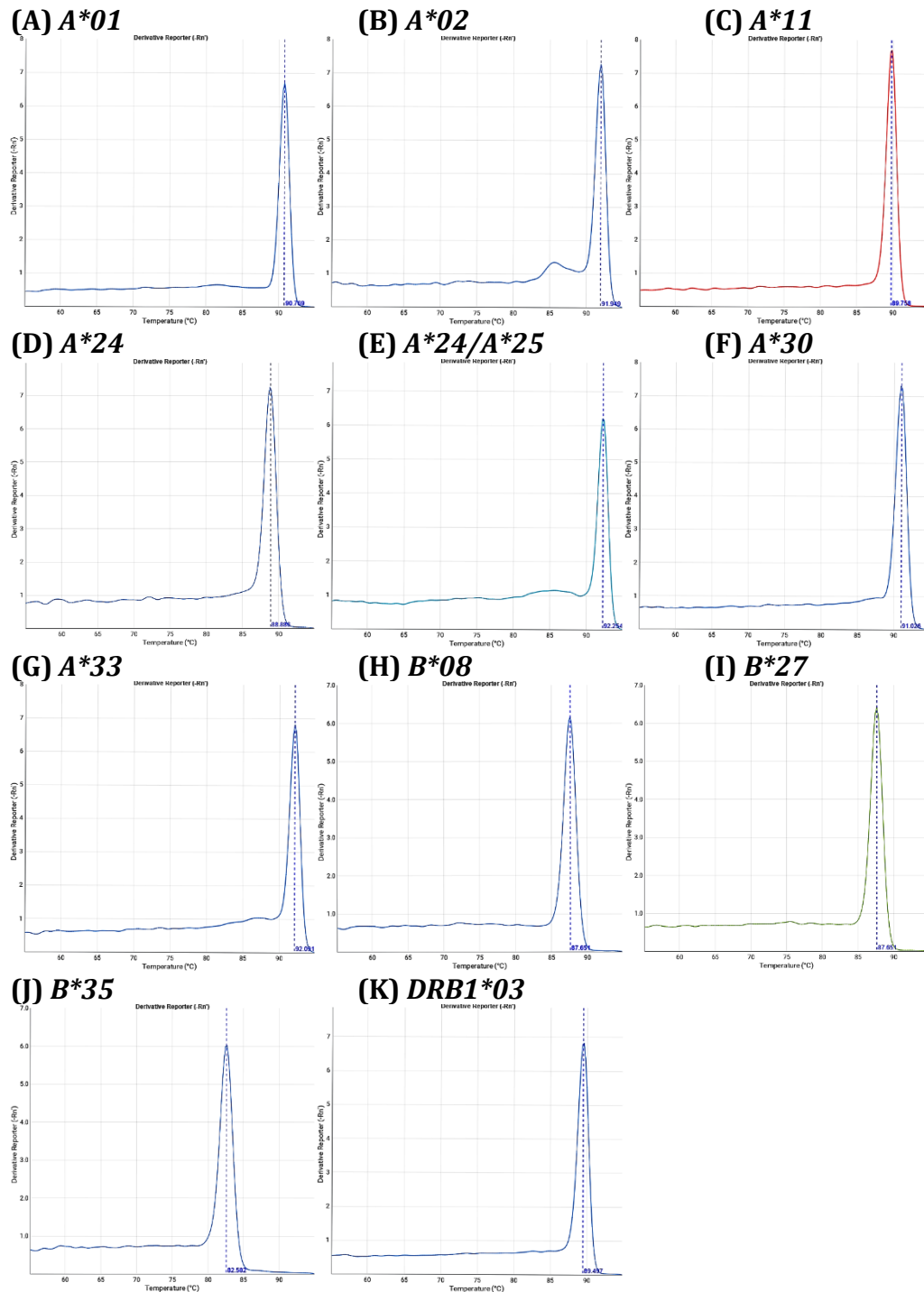
In silico HLA primer specificity testing was previously done by blasting the primer sequences on DNA templates for the target genes using the NCBI's Primer-BLAST online tool by colleagues in the Netherlands. Sequence homology between some HLA genes meant some primers showed *in silico* predicted cross-reactivity with other allele groups. Primer specificity was therefore further tested by setting up q-PCR reactions using a panel of samples both positive and negative for the target of interest (see **M&M section 2.7**).

Cross reactivity was defined as the presence of a T_m that fell within the range of the target of interest. The T_m of positive reactions and the cross-reactive alleles identified for each HLA primer pair, together with the *in silico* predicted cross-reactivity, are given in **Table 6.2**. Primers targeting *HLA-A*01*, *-A*11*, *-B*08* and *-B*35* were predicted to be cross-reactive and this was confirmed *in vitro* using the donor panel. No additional cross-reactivities or false positives were identified from *in vitro* testing. For all future experiments, primers that showed cross-reactivity were only used if the CB, or the male platelet donors used for standard curve generation, had no source of the cross-reactive allele.

Table 6.2 HLA q-PCR primer specificity. *In silico* primer specificity was evaluated by blasting the primer sequences on DNA templates for the target genes using the NCBI's Primer-BLAST online tool by colleagues in the Netherlands. Primer specificity was tested *in vitro* by setting up q-PCR reactions against a panel of HLA typed male platelet donors positive or negative for the target of interest. Melting temperature (T_m) is shown as the mean $\pm$ standard deviation for the expected positive reactions.

Target	Mean T_m (°C)	Cross reactivity	
		In silico	q-PCR
HLA-A			
*A*01*	90.80 $\pm$ 0.10	*A*11, A*36*	*A*11*
*A*02*	91.87 $\pm$ 0.10	-	-
*A*11*	89.82 $\pm$ 0.06	*A*02, A*24*	*A*02, A*24*
*A*24*	88.95 $\pm$ 0.04	-	-
*A*24/A*25*	91.93 $\pm$ 0.07	-	-
*A*30*	90.87 $\pm$ 0.11	-	-
*A*33*	92.27 $\pm$ 0.06	-	-
HLA-B			
*B*08*	87.36 $\pm$ 0.08	*B*41, B*42*	*B*41, B*42*
*B*27*	88.55 $\pm$ 0.08	-	-
*B*35*	82.73 $\pm$ 0.10	*B*08, B*78*	*B*08*
HLA-DRB1			
*DRB1*03*	89.10 $\pm$ 0.12	-	-

6.3.2 HLA primer concentration

The next step of the optimisation process was the selection of the appropriate primer concentration based on the efficiencies of the q-PCR reactions. Standard curves were generated from serial dilutions created from artificial spiking experiments, as described in **M&M section 2.7**.

HLA standard curves were tested at a 4.5 pmol, 3.0 pmol and 1.5 pmol of forward and reverse primer per reaction as a minimum and additional concentrations were tested if necessary. The amplification efficiencies and standard curve parameters for each primer pair concentration tested for each HLA target are shown in **Table 6.3**. Primer concentration that gave an efficiency between 90 and 110% were selected as the optimum concentrations for future experiments. These were as follows: *A*01*, 3.0 pmol; *A*02*, 1.5 pmol; *A*11*, 3.0 pmol; *A*24*, 4.5 pmol; *A*24/A*25*, 2.25 pmol; *A*30*, 1.5 pmol; *A*33*, 3.0 pmol; *B*08*, 2.25 pmol; *B*27*, 4.5 pmol; *B*35*, 1.5 pmol, *DRB1*03*, 3.0 pmol.

Table 6.3 Amplification efficiencies from q-PCR standard curves set-up using a variable primer concentration. Standard curves were produced from artificial spiking experiments whereby DNA positive for the HLA target of interest was diluted in DNA negative for the target. Serial 1:10 dilutions over five logs (100%, 10%, 1%, 0.1% and 0.01%) were pipetted from a starting quantity of 200ng and an additional 0.05% dilution was also included. q-PCR reactions for each dilution and target were set up in duplicate containing a variable amount of forward and reverse HLA primer. **Red** indicates amplification efficiency closest to 100% and thus the optimal primer concentration selected for future experiments.

Target	Amplification efficiency (%) at each primer concentration tested			
	4.5 pmol	**3.0 pmol**	**2.25 pmol**	**1.5 pmol**
HLA-A				
*A*01*	89	**90**	-	76
*A*02*	83	95	**-**	**98**
*A*11*	95	**97**	-	92
*A*24*	**98**	122	-	162
*A*24/A*25*	100	103	**98**	95
*A*30*	104	91	-	**94**
*A*33*	95	**96**	-	95
HLA-B				
*B*08*	110	99	**96**	147
*B*27*	**94**	87	-	80
*B*35*	111	112	-	**104**
HLA-DRB1				
*DRB1*03*	98	**96**	-	106

6.3.3 HLA assay performance

The sensitivities of the q-PCR assays were determined from the linear dynamic range (LDR) and the lowest dilution whereby four out of five replicates of expected positives were detected as positive in the q-PCR reaction. A standard

curve using artificial chimeric mixes of 100%, 10%, 1%, 0.1%, 0.05%, 0.01%, 0.005% was set up for each target. The sensitivity of each assay are given in **Table 6.4**. Each HLA specific q-PCR assay had a sensitivity of LDR of 0.01% and at least 4 of the 5 replicates were positive in the q-PCR reaction.

Table 6.4 Linear dynamic range (LDR) and number of positive replicates for q-PCR standard curves assays. Standard curves were produced from artificial spiking experiments whereby DNA positive for the HLA target of interest was diluted in DNA negative for the target. Serial dilutions (10%, 1%, 0.1%, 0.05%, 0.01% and 0.005%) were pipetted from a starting quantity of 200ng. q-PCR reactions for each dilution and target were set up with five replicates. LDR was the last point where the curve maintained linearity, defined as a coefficient of determination (R^2) $\geq$ 0.99.

Target	LDR (%)	Positive replicates
HLA-A		
A*01	0.01	5/5
A*02	0.01	5/5
A*11	0.01	4/5
A*24	0.01	4/5
A*24/A*25	0.01	5/5
A*30	0.01	4/5
A*33	0.01	4/5
HLA-B		
B*08	0.01	4/5
B*27	0.01	5/5
B*35	0.01	5/5
HLA-DRB1		
DRB1*03	0.01	5/5

Intra-assay precision was evaluated from the standard curve correlation and standard deviation of replicates. Representative amplification plots and standard curves of triplicate 1:10 serial dilutions over five logs for each HLA specific q-PCR assay are shown in **Figure 6.3** and **Figure 6.4** respectively. The mean C_q values and standard deviations for each standard curve are shown in **Table 6.5** and the standard curve properties are given in **Table 6.6.** Each standard curve showed excellent intra-assay precision. All standard curves showed an $R^2 \geq 0.99$ and the standard deviation of individual replicates for each experiment were within the acceptable range.

Figure 6.3 Representative HLA q-PCR amplification plots for standard curves. (A) *A*01*, **(B)** *A*02*, **(C)** *A*11*, **(D)** *A*24*, **(E)** A*25/A*24, **(F)** *A*30,* **(G)** *A*33,* **(H)** *B*08,* **(I)** *B*27,* **(J)** *B*35* and **(K)** *DRB1*03.* Standard curves were produced from artificial spiking experiments whereby DNA positive for the HLA target of interest was diluted in DNA negative for the target. Serial 1:10 dilutions over five logs (100%, 10%, 1%, 0.1% and 0.01%) were pipetted from a starting quantity of 200ng and set up in triplicate.

(A) *A*01*

(B) *A*02*

(C) *A*11*

(D) *A*24*

(E) *A*24/A*25*

(F) *A*30*

(G) *A*33*

(H) *B*08*
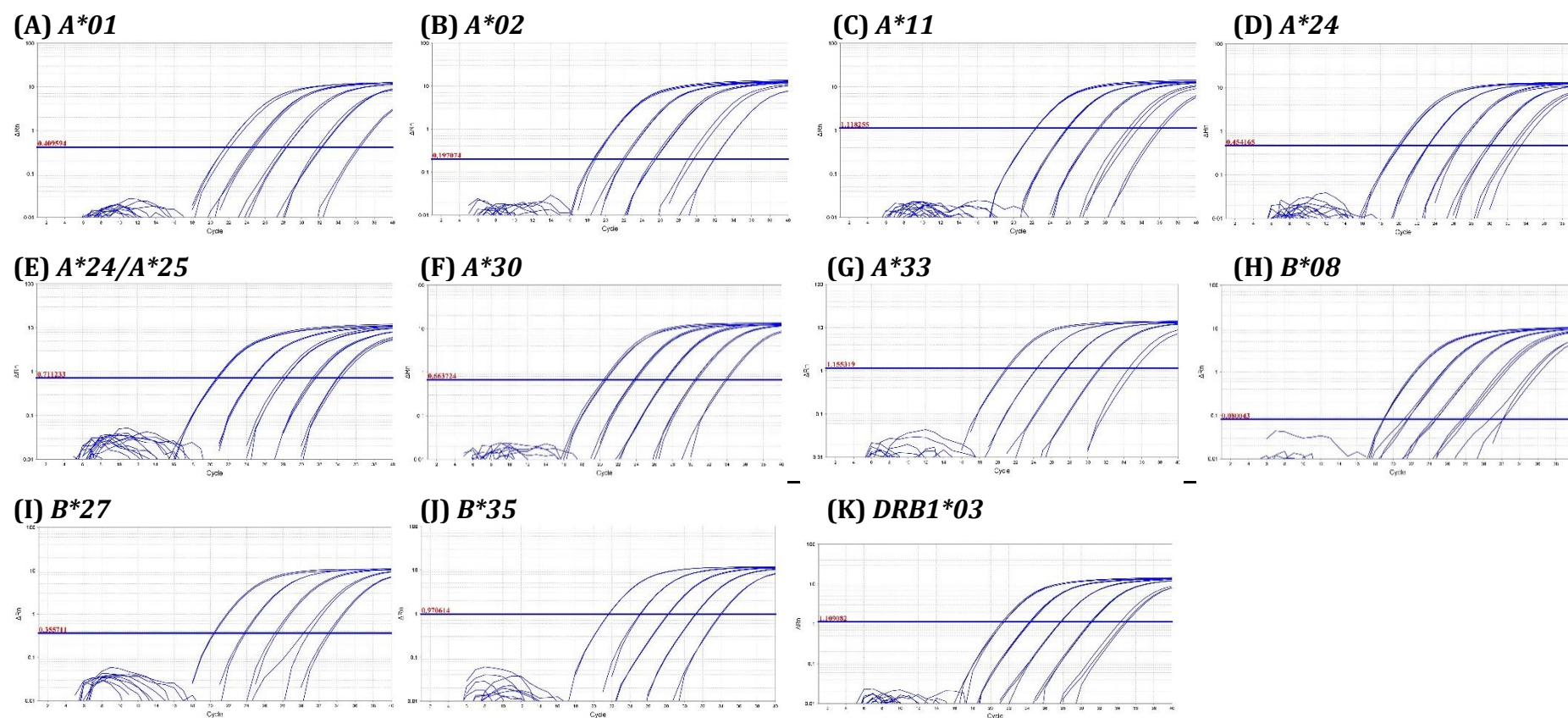
(I) *B*27*

(J) *B*35*

(K) *DRB1*03*

Figure 6.4 Representative HLA q-PCR standard curves. (A) *A*01*, **(B)** *A*02*, **(C)** *A*11*, **(D)** *A*24*, **(E)** *A*24/A*25*, **(F)** *A*30*, **(G)** *A*33,* **(H)** *B*08,* **(I)** *B*27,* **(J)** *B*35* and **(K)** *DRB1*03.* Standard curves were produced from artificial spiking experiments whereby DNA positive for the HLA target of interest was diluted in DNA negative for the target. Serial 1:10 dilutions over five logs (100%, 10%, 1%, 0.1% and 0.01%) were pipetted from a starting quantity of 200ng and an additional 0.05% dilution was also included. q-PCR reactions for each dilution and target were set up in triplicate.

(A) *A*01*
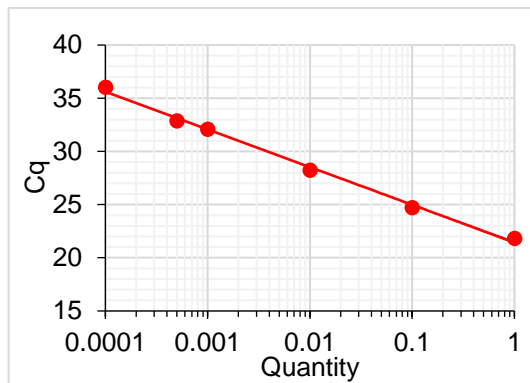
(B) *A*02*
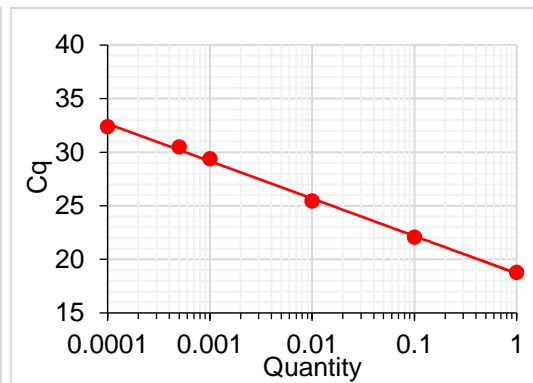
(C) *A*11*
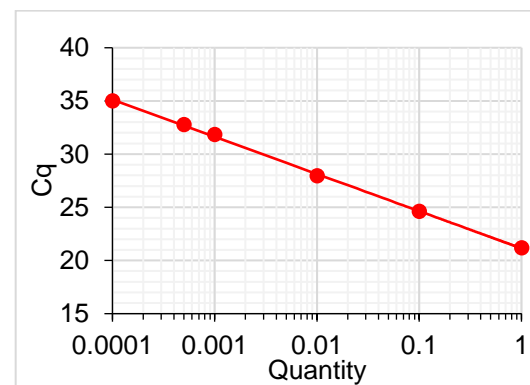
(D) *A*24*
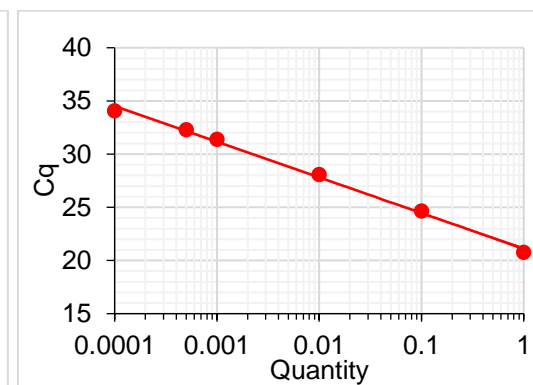
(E) *A*24/A*25*
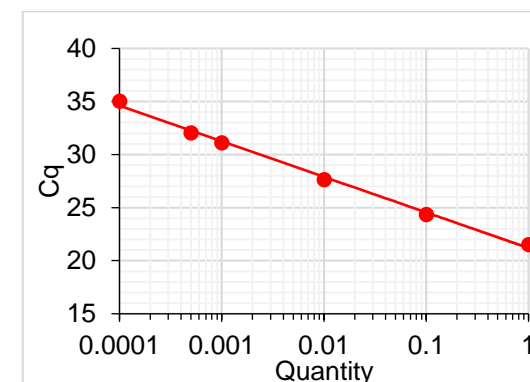
(F) *A*30*
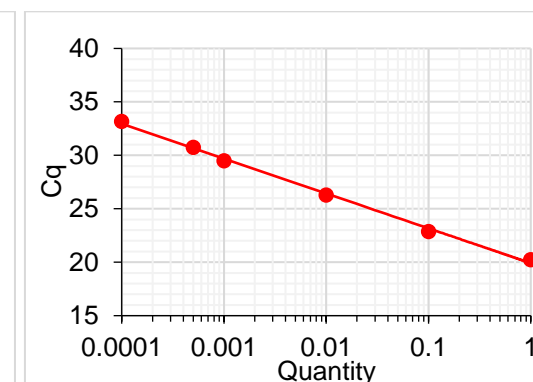
Figure 6.4 continued

(G) *A*33*
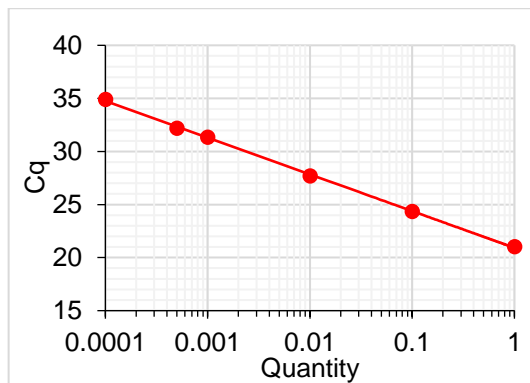
(H) *B*08*
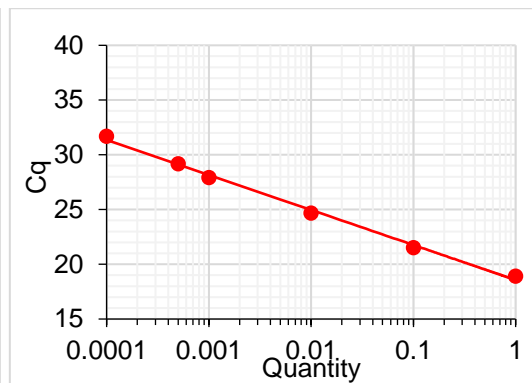
(I) *B*27*
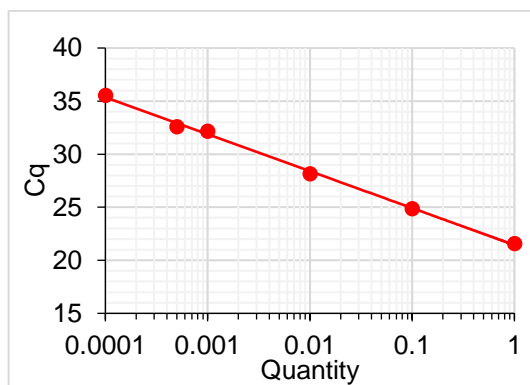
(J) *B*35*
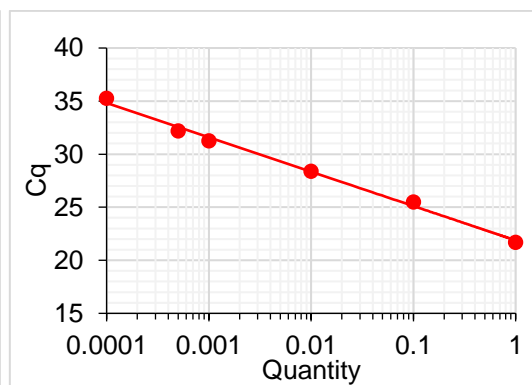
(K) *DRB1*03*
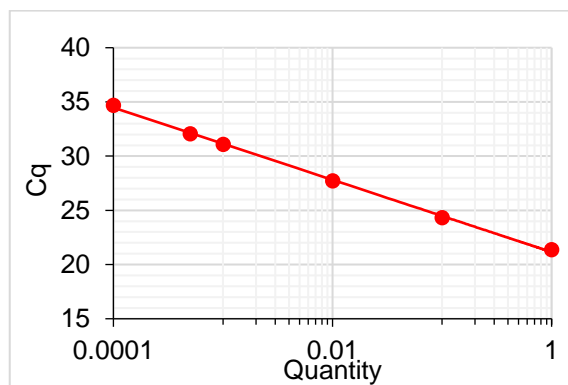
Table 6.5 HLA q-PCR quantitation cycle (C_q) results for representative standard curves. Standard curves were produced from artificial spiking experiments whereby DNA positive for the HLA target of interest was diluted in DNA negative for the target. Serial 1:10 dilutions over five logs (100%, 10%, 1%, 0.1% and 0.01%) were pipetted from a starting quantity of 200ng and an additional 0.05% dilution was also included. q-PCR reactions for each dilution and target were set up in triplicate. C_q values are shown as the mean ± standard deviation (S.D.) for three replicates.

Target	Mean C_q ± S.D. for each target quantity					
	1	0.1	0.01	0.001	0.0005	0.0001
HLA-A						
*A*01*	21.831 ± 0.276	24.712 ± 0.188	28.232 ± 0.248	32.092 ± 0.265	32.862 ± 0.333	36.033 ± 0.538
*A*02*	18.777 ± 0.120	22.071 ± 0.163	25.441 ± 0.148	29.395 ± 0.397	30.506 ± 0.296	32.373 ± 0.817
*A*11*	21.179 ± 0.102	24.622 ± 0.023	27.937 ± 0.171	31.837 ± 0.640	32.753 ± 0.420	34.998 ± 0.948
*A*24*	21.523 ± 0.036	24.365 ± 0.068	27.643 ± 0.097	31.128 ± 0.520	32.060 ± 0.298	35.034 ± 0.929
*A*24/A*25*	20.758 ± 0.086	24.652 ± 0.065	28.063 ± 0.208	31.385 ± 0.193	32.296 ± 0.361	34.067 ± 0.187
*A*30*	20.240 ± 0.143	22.876 ± 0.071	26.299 ± 0.097	29.492 ± 0.140	30.751 ± 0.312	33.183 ± 0.419
*A*33*	21.024 ± 0.099	24.347 ± 0.023	27.709 ± 0.059	31.347 ± 0.093	32.186 ± 0.171	34.912 ± 0.516
HLA-B						
*B*08*	18.938 ± 0.043	21.521 ± 0.223	24.672 ± 0.161	27.937 ± 0.269	29.175 ± 0.475	31.691 ± 0.826
*B*27*	21.586 ± 0.036	24.861 ± 0.037	28.152 ± 0.170	32.172 ± 0.861	32.588 ± 0.340	35.540 ± 0.998
*B*35*	21.703 ± 0.042	25.505 ± 0.226	28.402 ± 0.021	31.249 ± 0.174	32.185 ± 0.293	35.259 ± 1.148
HLA-DRB1						
*DRB1*03*	21.354 ± 0.115	24.323 ± 0.092	27.716 ± 0.043	31.074 ± 0.073	32.053 ± 0.126	34.691 ± 0.373

Table 6.6 Representative HLA q-PCR standard curves properties. Standard curves were produced from artificial spiking experiments whereby DNA positive for the HLA target of interest was diluted in DNA negative for the target. Serial 1:10 dilutions over five logs (100%, 10%, 1%, 0.1% and 0.01%) were pipetted from a starting quantity of 200ng and an additional 0.05% dilution was also included. q-PCR reactions for each dilution and target were set up in triplicate.

Target	Slope	Intercept	R^2
HLA-A			
A*01	-3.643	21.132	0.993
A*02	-3.484	18.690	0.994
A*11	-3.495	21.140	0.992
A*24	-3.352	21.194	0.990
A*24/A*25	-3.352	21.108	0.995
A*30	-3.365	20.497	0.998
A*33	-3.424	21.414	0.997
HLA-B			
B*08	-3.199	18.564	0.990
B*27	-3.478	21.439	0.991
B*35	-3.184	20.495	0.999
HLA-DRB1			
DRB1*03	-3.330	21.153	0.998

6.3.4 HCK optimisation and assay performance

HCK was chosen as the endogenous control for calculating the total starting template present in a reaction. The HCK primers were shown to be specific for HCK *in vitro* **(Figure 6.5A)**. HCK was optimised to a primer-limiting concentration and after a trial of 4 pmol (E = 119%), 2 pmol (E = 116%), 1 pmol (E = 97%) and 0.5 pmol (E = 70%) of each primer per reaction, 1 pmol was selected for all future experiments. At this concentration, amplification was

exponential **(Figure 6.5B-C)** with excellent intra-assay precision **(Table 6.7)**.

The standard curve properties were as follows: slope, -3.378; intercept, 23.209

and $R^2 \geq 0.990$.

Figure 6.5 Representative HCK q-PCR A) dissociation curve, B) amplification plot and c) standard curve. Standard curves were produced from artificial spiking experiments whereby DNA positive for the HLA target of interest was diluted in DNA negative for the target. Male donors were serially diluted six times by 1:2 in H_2O from a starting quantity of 250 ng and q-PCR reactions were set up in duplicate for each dilution.

(A)

(B)

(C)
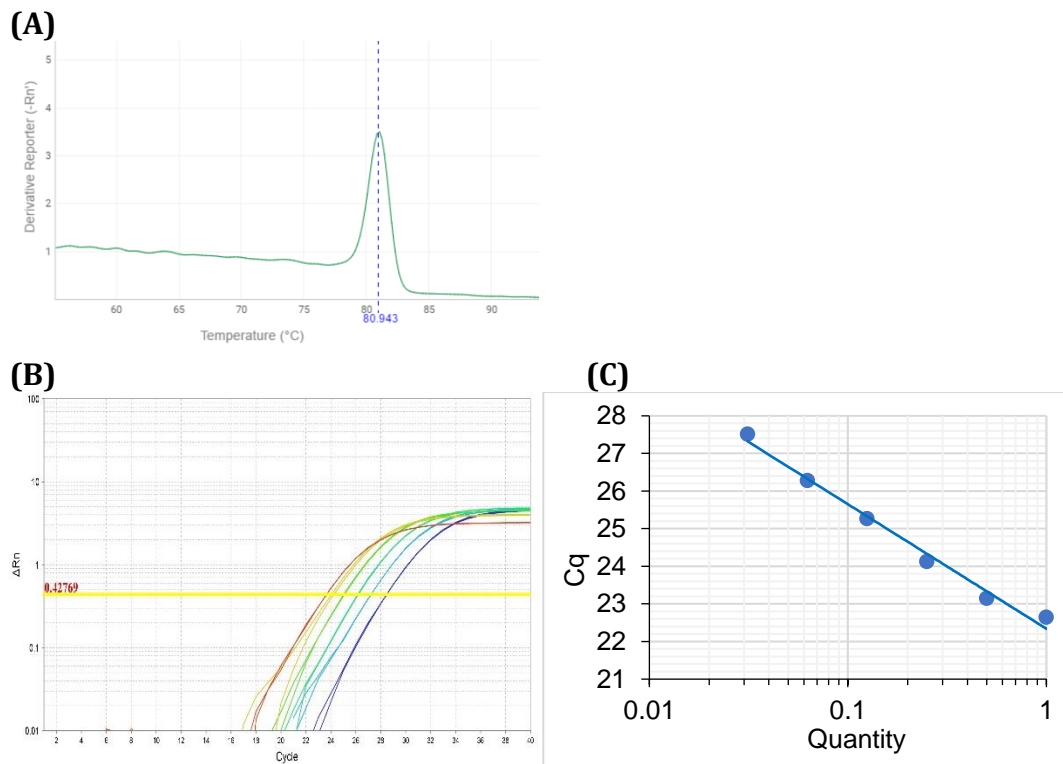
Table 6.7 HCK q-PCR quantitation cycle (C_q) results for the representative standard curve. Standard curves were produced from artificial spiking experiments whereby DNA positive for the HLA target of interest was diluted in DNA negative for the target. Male donors positive were serially diluted six times by 1:2 in H_2O from a starting quantity of 250 ng and q-PCR reactions were set up in duplicate for each dilution. C_q values are shown as the mean ± standard deviation (S.D.).

Quantity	Mean C_q ± S.D.
1	22.644 ± 0.010
0.5	23.152 ± 0.176
0.25	24.124 ± 0.035
0.125	25.264 ± 0.037
0.0625	26.283 ± 0.014
0.03125	27.515 ± 0.022

6.4 MMc in clinical grade CB samples

6.4.1 Detection and quantification of MMc in CB by q-PCR

The optimised HLA specific q-PCR assays were used to test for the presence of MMc in a cohort of clinical grade CBUs. MMc was detected by testing for the presence of NIMA DNA in the CB DNA. The first step was to evaluate mothers for the presence of one or more HLA alleles with a primer available for targeting. There were 175 CB-maternal pairs evaluated and nine mothers did not possess one or more of the HLA targets with primers available. The HLA types of the remaining 166 CB and mothers were compared to identify the NIMA. The maternal HLA allele was either not the CB NIMA (i.e. it was the IMA), or it was not possible to identify whether it was the NIMA (i.e. non-informative due to maternal homozygosity or 0 MM to the CB), in a further 70 cases and these were therefore unable to be evaluated for the presence of MMc. The frequency of maternal HLA alleles for each of the q-PCR targets and how often these were the

IMA, NIMA or non-informative are shown in **Table 6.8.** Of the targets evaluated, maternal *HLA-A*01* and *-A*02* were most often non-informative for NIMA (23% and 28%, respectively). There were 96 CB samples with an informative NIMA HLA that was available as a q-PCR assay target and these were tested for MMc using the HLA specific q-PCR assay.

Samples were tested for MMc by targeting the NIMA at *HLA-A* (n= 69), *-B* (n= 23) or *–DRB1* (n= 4) **(Table 6.8)**. Twenty-seven percent (n= 26) were positive for MMc and 73% (n= 70) were negative for MMc. In those testing positive, the proportion of maternal DNA in the CBU varied from 0.01% to 2.3% and the median quantity of those positive was 0.05% **(Figure 6.6).**

Table 6.8 Informativity of maternal HLA alleles for NIMA and the incidence of MMc above 0.01% in CB. A total of 175 CB-maternal pairs were evaluated for the presence of an allele that was informative NIMA for which a q-PCR assay was available. MMc was quantified in 96/175 CB samples by a q-PCR absolute quantification method that calculates the quantity of NIMA HLA template from the quantity of the reference, HCK and to a sensitivity of 0.01%.

Target	Mother	IMA/IPA			MMc		
		Non-informative	IMA	NIMA	Total tested	Negative	Positive
A*01	66	15 (23)	25 (38)	26 (39)	14	12 (86)	2 (14)
A*02	74	21 (28)	29 (39)	24 (32)	22	19 (86)	3 (14)
A*11	28	4 (14)	7 (25)	17 (61)	12	5 (42)	7 (58)
A*23	5	0 (0)	1 (20)	4 (80)	2	2 (100)	0 (0)
A*24	28	3 (11)	16 (57)	9 (32)	9	6 (33)	3 (66)
A*25	5	0 (0)	4 (80)	1 (20)	0	-	-
A*30	12	2 (17)	3 (25)	7 (58)	7	7 (100)	0 (0)
A*33	11	2 (18)	6 (55)	3 (27)	3	3 (100)	0 (0)
B*08	44	3 (7)	20 (45)	21 (48)	12	5 (42)	7 (58)
B*27	10	0 (0)	4 (40)	6 (60)	3	3 (100)	0 (0)
B*35	29	6 (21)	12 (41)	11 (38)	8	4 (50)	4 (50)
DRB1*03	53	9 (17)	19 (36)	25 (47)	4	4 (100)	0 (0)
Total	**-**	**-**	**-**	**-**	**96**	**70 (73)**	**26 (27)**

Figure 6.6 Quantity of MMc in CB. MMc was quantified in 96 CB samples using a q-PCR absolute quantification method that calculates the quantity of NIMA HLA template from the quantity of the reference, HCK and to a sensitivity of 0.01%. The means of triplicate HLA and duplicate HCK q-PCR were used to determine the proportion of MMc in the samples.
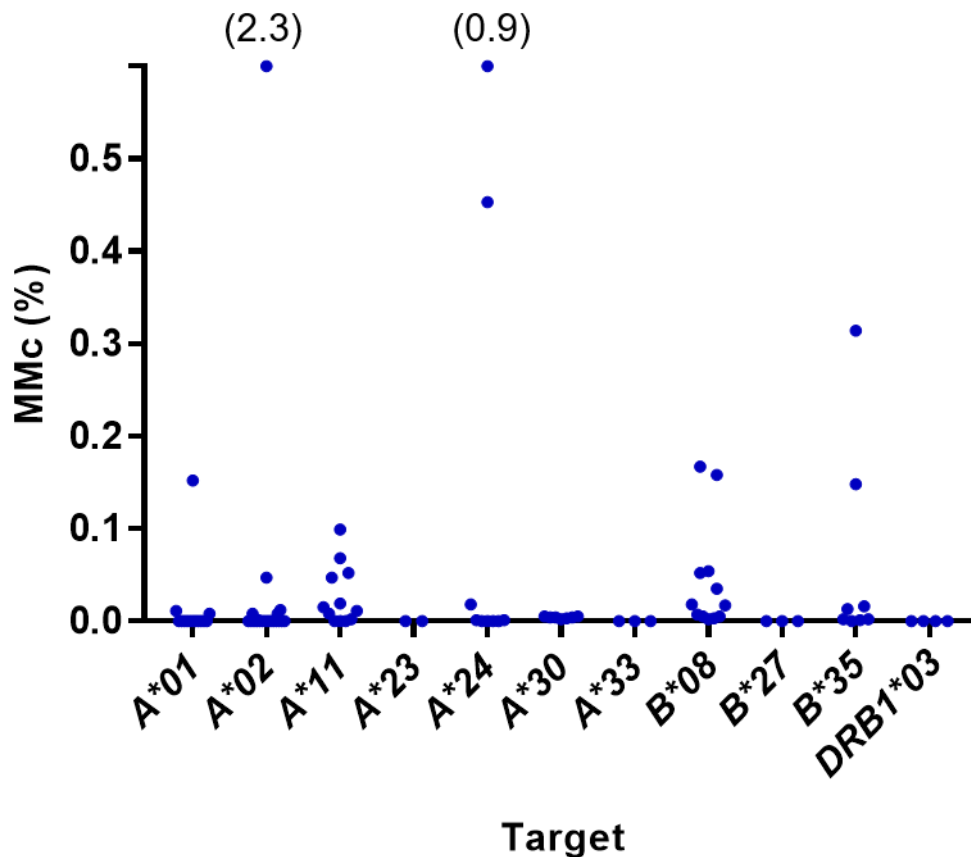
6.4.2 Characteristics of CBUs and mothers

The main characteristics of the CBU and maternal pairs included in the study are given in **Table 6.9**. Demographics were not available for all those tested. In those where data was available, the median age of mothers was 31, the majority entered labour naturally (*n*= 21, 88%) and experienced vaginal delivery as opposed to a caesarean section (*n*= 36, 77%). The median year of birth was 2001, the majority of new-borns were Caucasian (*n*= 55, 65%) and the majority were delivered at 39 weeks or later (*n*= 58, 81%). No significant differences in

maternal and new-born characteristics were identified between the CBUs positive or negative for MMc, except gestational age. A smaller proportion of new-borns were delivered before 39 weeks in the MMc negative group (n= 7, 13%) compared to the positive group (n= 7, 35%; p= 0.03).

Table 6.9 Characteristics of mothers and their CBUs. Percentages are given in parenthesis.

	Total	MMc		
		Positive	**Negative**	**P**
Mother				
Age (years) (n= 40)				0.70
Mean	31	30	31	
Range	19 - 42	25 – 37	19 - 42	
Gravida (n= 13)				0.07
Primi	5 (38)	1 (50)	4 (36)	
Multi	8 (62)	1 (50)	7 (64)	
Induction (n= 24)				0.48
No	21 (88)	3 (100)	18 (86)	
Yes	3 (12)	0 (0)	3 (14)	
Method of delivery (n= 47)				0.73
Vaginal	36 (77)	8 (73)	28 (78)	
Caesarean section	11 (23)	3 (27)	8 (22)	
New-born				
Year of birth (n= 96)				0.99
Median	2001	2001	2001	
Range	1996 - 2012	1996 - 2009	1996 - 2012	
Gender (n= 96)				0.83
Male	50 (52)	14 (54)	36 (51)	
Female	46 (48)	12 (46)	34 (49)	
Ethnicity (n= 85)				0.25
Caucasian	55 (65)	17 (71)	38 (62)	
Asian	17 (20)	6 (25)	11 (18)	
Black	5 (6)	0 (0)	5 (8)	
Mixed	8 (9)	1 (4)	7 (11)	
Gestational age (n= 72)				**0.03**
Pre- and early-term ($\leq$38 weeks)	14 (19)	7 (35)	7 (13)	
Term or longer ($\geq$39 weeks)	58 (81)	13 (65)	45 (87)	
Total nucleated cell count (x10^7) (n= 91)				0.21
Average	189	168	189	
Range	48 - 589	57 - 303	48 - 589	

6.4.3 Association of MMc with maternal-foetal HLA allele frequencies and histocompatibility

The *HLA-A*, *-B*, *-C* and *-DRB1* IMA, IPA and NIMA allele frequencies of maternal-foetal pairs positive or negative for MMc were compared. *HLA-A* NIMA allele frequencies significantly differed between MMc positive and MMc negative groups (*p*= 0.033) and comparison of column proportions revealed this difference was due to a higher frequency of NIMA *HLA-A*11* in the MMc positive group. However, significant differences in *HLA-A* frequencies between the groups were not observed for the CBU IMA (*p*= 0.598) or IPA alleles (*p*= 0.744). Maternal-foetal pairs positive or negative for MMc showed comparable HLA allele frequencies at *HLA-B* (NIMA: *p*= 0.499, IMA: *p*= 0.971, IPA: *p*= 0.294), *-C* (NIMA: *p*= 0.722, IMA: *p*= 0.920, IPA: *p*= 0.839) and *-DRB1* (NIMA: *p*= 0.393, IMA: *p*= 0.836, IPA: *p*= 0.265).

Table 6.10 *HLA-A*, *-B*, *-C* and *-DRB1* IMA and IPA and NIMA allele frequencies of maternal-foetal pairs positive or negative for MMc

	NIMA			IMA			IPA		
	MMc neg	MMc pos	Total	MMc neg	MMc pos	Total	MMc neg	MMc pos	Total
HLA-A									
A*01	27	21	25	14	26	17	19	26	20
A*02	30	16	27	22	16	20	13	32	17
A*03	2	11	4	16	11	14	19	5	16
A*11	8	37	14	6	0	5	8	5	7
A*23	3	0	2	0	5	1	2	0	1
A*24	9	16	11	11	11	11	6	11	7
A*25	0	0	0	2	5	2	2	0	1
A*26	0	0	0	5	5	5	5	5	5
A*29	2	0	1	6	5	6	2	0	1
A*30	11	0	8	5	0	4	3	0	2
A*31	3	0	2	5	0	4	2	0	1

	NIMA			IMA			IPA		
	MMc neg	MMc pos	Total	MMc neg	MMc pos	Total	MMc neg	MMc pos	Total
*A*32*	2	0	1	2	0	1	3	0	2
*A*33*	5	0	4	2	5	2	8	5	7
*A*68*	0	0	0	5	11	6	11	11	11
*A*74*	0	0	0	2	0	1	0	0	0
HLA-B									
*B*07*	9	0	7	11	13	11	15	8	13
*B*08*	18	33	22	11	13	11	9	8	9
*B*13*	3	4	3	2	4	2	0	4	1
*B*14*	0	4	1	3	0	2	6	0	4
*B*15*	5	8	6	8	13	9	11	8	10
*B*18*	5	0	3	3	8	4	5	0	3
*B*27*	9	0	7	3	0	2	5	8	6
*B*35*	11	13	11	11	8	10	5	4	4
*B*37*	2	0	1	0	0	0	0	4	1
*B*38*	0	0	0	3	4	3	3	4	3
*B*39*	0	0	0	3	0	2	0	0	0
*B*40*	5	13	7	8	8	8	9	4	8
*B*41*	0	0	0	2	0	1	0	0	0
*B*42*	0	0	0	0	0	0	2	0	1
*B*44*	8	13	9	18	25	20	12	8	11
*B*45*	2	0	1	2	0	1	0	0	0
*B*48*	3	0	2	0	0	0	0	0	0
*B*49*	2	0	1	0	0	0	2	8	3
*B*50*	2	0	1	0	0	0	0	4	1
*B*51*	5	4	4	5	0	3	0	4	1
*B*52*	2	0	1	3	0	2	5	8	6
*B*53*	2	0	1	2	0	1	5	0	3
*B*55*	2	0	1	5	0	3	0	4	1
*B*56*	0	4	1	0	0	0	2	0	1
*B*57*	6	4	6	2	4	2	5	4	4
*B*58*	5	0	3	0	0	0	3	4	3
HLA-C									
*C*01*	2	6	3	5	6	5	2	6	3
*C*02*	5	0	3	2	0	2	5	0	3
*C*03*	17	6	14	17	6	14	15	12	14
*C*04*	20	18	19	10	6	9	15	6	12
*C*05*	7	6	7	12	6	10	15	6	12
*C*06*	12	0	9	2	12	5	2	18	7

	NIMA			IMA			IPA		
	MMc neg	MMc pos	Total	MMc neg	MMc pos	Total	MMc neg	MMc pos	Total
*C*07*	22	41	28	24	29	26	17	24	19
*C*08*	5	6	5	2	0	2	7	6	7
*C*12*	2	6	3	10	18	12	10	12	10
*C*14*	2	6	3	0	0	0	0	0	0
*C*15*	2	6	3	5	6	5	5	6	5
*C*16*	2	0	2	10	12	10	5	6	5
*C*17*	0	0	0	0	0	0	2	0	2
HLA-DRB1									
*DRB1*01*	11	9	11	5	13	7	8	9	8
*DRB1*03*	31	35	32	13	9	12	15	4	12
*DRB1*04*	15	13	14	20	13	18	13	17	14
*DRB1*07*	13	9	12	16	26	19	8	22	12
*DRB1*08*	0	0	0	5	0	4	8	0	6
*DRB1*09*	0	4	1	2	0	1	2	0	1
*DRB1*10*	0	0	0	3	0	2	0	0	0
*DRB1*11*	7	9	7	7	4	6	11	13	12
*DRB1*12*	3	0	2	3	4	4	3	0	2
*DRB1*13*	7	13	8	7	4	6	13	4	11
*DRB1*14*	2	9	4	3	0	2	2	13	5
*DRB1*15*	11	0	8	15	17	15	16	17	17
*DRB1*16*	0	0	0	2	9	4	0	0	0

It was reasoned that maternal-foetal compatibility could be an important factor in MMc and this relationship was investigated. The mother was deemed compatible from the perspective of the foetus (HvG direction) if none of the maternal alleles differed in the foetus (the mother was homozygous or a 0 MM to the foetus). The foetus was deemed compatible from the perspective of the mother (GvH direction) if none of the foetal alleles different in the mother (the foetus was homozygous or a 0 MM to the mother). Results are shown in **Table 6.11.** Maternal compatibility from the foetal perspective at *HLA-A* (p= 0.096), *-B* (p= 0.970), *-C* (p= 0.610) or *-DRB1* (p= 0.176) was not significantly associated

with MMc. Similarly, foetal compatibility from the maternal perspective at *HLA-A* (*p*= 0.091), *-B* (*p*= 0.957), *-C* (*p*= 0.783) or *-DRB1* (*p*= 0.982) was not significantly associated with MMc.

At the maternal-foetal interface, there is a two-way interaction between the mother and foetus without mutual rejection. Bidirectional compatibility, defined as dual compatibility from each other's perspective (by only including 0 MM between the mother and foetus) was therefore also evaluated for an association with MMc. Bidirectional compatibility at *HLA-B*, *-C* and *-DRB1* had no effect on the incidence of MMc. Intriguingly, however, bidirectional compatibility at *HLA-A* was significantly associated with an increased incidence of MMc (OR, 3.900; 95% CI, 1.075 - 14.144; *p*= 0.029).

Table 6.11 CBU-maternal HLA compatibility for those positive and negative for MMc. Percentages are given in parenthesis.

	Number (%)			Statistics	
	Positive	**Negative**	**Total**	**OR (95% CI)**	***P***
Mother compatible from foetus' perspective					
HLA-A	6/26 (23.1)	7/70 (10.0)	13/96 (13.5)	2.700 (0.813 - 8.972)	0.096
HLA-B	2/26 (7.7)	5/67 (7.5)	7/93 (7.5)	1.033 (0.188 - 5.691)	0.970
HLA-C	6/19 (31.6)	13/51 (25.5)	19/70 (27.1)	1.349 (0.425 - 4.279)	0.610
HLA-DRB1	3/26 (11.5)	19/70 (27.1)	22/96 (22.9)	0.350 (0.094 - 1.302)	0.106
Foetus compatible from mother's perspective					
HLA-A	10/26 (38.5)	15/70 (21.4)	25/96 (26.0)	2.292 (0.865 - 6.074)	0.091
HLA-B	3/26 (11.5)	8/67 (11.9)	11/93 (11.8)	0.962 (0.234 - 3.947)	0.957
HLA-C	7/19 (36.8)	17/51 (33.3)	24/70 (34.3)	1.167 (0.389 - 3.501)	0.783
HLA-DRB1	6/26 (23.1)	16/70 (22.9)	22/96 (22.9)	1.013 (0.348 - 2.950)	0.982
Bidirectional compatibility between foetus and mother					
HLA-A	6/26 (23.1)	5/70 (7.1)	11/96 (11.6)	3.900 (1.075 - 14.144)	**0.029**
HLA-B	1/26 (3.8)	1/67 (1.5)	2/93 (2.2)	2.640 (0.159 - 43.840)	0.483
HLA-C	2/19 (10.5)	9/51 (17.6)	11/70 (15.7)	0.559 (0.107 - 2.809)	0.467
HLA-DRB1	3/26 (11.5)	8/70 (11.4)	11/96 (11.5)	1.011 (0.247 - 4.143)	0.988

6.5 Discussion

The aim of this chapter was to develop a sensitive q-PCR assay for the detection and quantification of MMc in CB, to determine the levels of MMc in transplanted CBUs and to investigate variables associated with its presence. This is a valuable tool that will enable a direct analysis of the influence of MMc in the CB graft on recipient clinical outcomes after transplantation in future work.

6.5.1 Successful development of a sensitive q-PCR assay that targets HLA polymorphisms according to MIQE Guidelines

HLA alleles were chosen as targets for chimaerism detection in the q-PCR assays because the HLA types of CBUs and mothers were readily available. This meant financially and time-costly prior genotyping of CBUs and mothers, to identify informative polymorphisms, was not required. Q-PCR was chosen because of its sensitivity and indeed, the assay was optimised and validated successfully to detect 0.01% of minor DNA within a major DNA component. The optimisation process included the determination of primer specificity, selection of target and endogenous control (HCK) primer concentration and determination of PCR efficiency. Each of the 11 primer pairs (*HLA-A*01, -A*02, -A*11, -A*24, -A*24/A*25, -A*30, -A*33, -B*08, -B*27, -B*35* and *-DRB1*03*) were tested for specificity *in vitro* by setting up q-PCR reactions using a panel of HLA typed male platelet donors. Analysis of dissociation curves confirmed the primers were specific. In cases where there was cross-reactivity (*HLA-A*01, -A*11, -B*08* and *-B*35*), the primers were only used in MMc quantification if there was no potential source of the cross-reactive allele from the mother or CBU. PCR efficiencies were determined by the generation of standard curves and after optimisation of the primer

concentration, each of the q-PCR assays demonstrated high amplification efficiencies (greater than 90%), with an $R^2 \geq 0.99$, indicating that the thermal cycling parameters were optimised. This also meant that each of the q-PCR assays were able to accurately and reliably quantify MMc across 5 logs of magnitude, to a sensitivity of 0.01%, demonstrated by the proportion of expected positive replicates testing positive in the assay and precision of the assay (standard deviation of replicates).

6.5.2   Detection of MMc in clinical grade CBUs

In this study, 27% (*n*= 26/96) of CBUs tested positive for NIMA HLA DNA in a q-PCR assay. Intriguingly, MMc was significantly associated with histocompatibility at *HLA-A* but only when compatibility was from the perspective of both the mother and foetus, i.e. in both the GvH and HvG direction (for example, if both the mother and foetus expressed HLA-A*01, *02). Class I compatibility may be important for enabling maternal cells to survive in the foetus due to the absence of a foetal class I mediated CD8^{+} CTL response directed against NIMA and indeed, MMc has been shown to induce allospecific regulation of the direct or semi-direct pathway of the CTL response (Burlingham*, et al* 1995, Moretta*, et al* 1999). However, several other studies have consistently demonstrated the lack of regulation of the direct pathway CTL response against NIMA (Akiyama*, et al* 2011, Hadley*, et al* 1990, Roelen*, et al* 1995).

The observation that MMc was not associated with histocompatibility in the HvG perspective yet was associated with bidirectional compatibility from both the mother and foetal perspective is intriguing and requires the assumption that the

maternal cells transferred are functioning. Some evidence is able to support this hypothesis. In CBT, maternal cells sensitised against foetal IPAs have been proposed to mediate a GvL response in CBT for patients with malignant disease (van Rood, *et al* 2012). Maternal renal grafts have also been associated with poor clinical outcomes, possibly because the tolerogenic NIMA effect has been out-weighted by an anti-IPA effector response elicited by passenger maternal lymphocytes, in what is termed the "NIMA paradox;" although the effects of passenger lymphocytes in renal grafts has not been extensively studied (Lim, *et al* 2016, Opelz 1990, van Rood and Claas 2000). Some awareness of the functional capabilities of chimeric cells can be gleaned from maternal cells isolated from children with SCID. These maternal microchimaeric cells have been shown to express HLA molecules and T cell activation markers (Muller, *et al* 2001) but do not seem to function *in vitro* when stimulated with alloantigen (Thompson, *et al* 1984), possibly due to a limited T cell receptor repertoire (Knobloch, *et al* 1991). However, MMc has been associated with GvHD in SCID patients, demonstrating functionality *in vivo* (Muller, *et al* 2001). Interestingly, GvHD does not develop in all SCID patients with detectable MMc, suggesting a regulatory response may control maternal engrafted lymphocytes.

Recent evidence from a prospective study in a living-related renal transplant population suggests pre-transplant immune regulation to NIMA was able to benefit clinical outcome of mother to child renal transplants *but only if regulation was reciprocated on the maternal (anti-IPA) side* (Jankowska-Gan, *et al* 2012). Why MMc was associated with bidirectional compatibility at *HLA-A* remains unclear. It has been hypothesised that maternal microchimaeric cells are

involved in the reprogramming of DCs towards a tolerogenic state and induce anergy via the indirect pathway, thus permitting the survival of maternal microchimaeric cells and amplification of MMc and tolerance (Bracamonte-Baran and Burlingham 2015). Whether *HLA-A* plays a specific role in MMc or was just the only locus to be statistically significant due to the heterogeneity of HLA and small sample size also remains unclear. Other studies have identified an association of MMc with compatibility at the class II antigens, *HLA-DRB1* and -*DQB1* but not at class I (Berry*, et al* 2004, Lambert*, et al* 2004). The reasons for these differences between studies are unclear but may be in part due to the heterogeneous nature of the HLA types present in the CBUs included in this study. HLA-DQB1 typing was unavailable for CBUs and mothers in this cohort. Analysis of maternal-foetal compatibility at the epitope level, as opposed to antigen compatibility, may also provide further information.

MMc was not detected in all CB donors and the factors associated with the presence of MMc remain unclear. NK cells play an important role during pregnancy, possibly mediated through the expression of killer immunoglobulin-like receptors (KIRs) that bind to HLA ligands and result in either inhibition or activation of a natural killer cell responses. Foetal-maternal KIR compatibility has been studied extensively from the perspective of maternal allorecognition of the foetus, with studies demonstrating a role in pre-eclampsia and recurrent miscarriage (Hiby*, et al* 2004, Parham and Moffett 2013). Foetal-maternal KIR compatibility could also play a role in the opposite direction: foetal allorecognition of the mother and MMc. Further work could therefore explore

frequencies and combinations of activating and inhibitory KIR genes and their ligands and the presence and levels of MMc.

The NIMA HLA detected could be cellular DNA or cell-free circulating DNA. Although studies of cell-free DNA in CB have not been published, mothers are known to harbour cell-free circulating DNA of foetal origin during pregnancy and recently this has been exploited for non-invasive antenatal testing of foetal genetic abnormalities (Benn 2016). Chimeric cell-free circulating DNA of donor origin has also been detected in the urine of solid organ recipients (Gielis, *et al* 2015). If NIMA HLA detected in q-PCR was of cellular origin, it is not known whether transfer of maternal cells to the foetus occurred during an active/passive transfer during pregnancy or whether the maternal cells entered the CB during labour or during the CB harvest.

Considerable evidence suggests the presence of MMc in CB occured during pregnancy. MMc has been detected in both healthy adults (Lambert, *et al* 2004) and pre-term foetuses (Mold, *et al* 2008). In this study, MMc was significantly associated with gestational age, with 50% (*n*= 7/14) of CBUs belonging to babies born at 38 weeks or less testing positive for MMc, compared to 22% (*n*= 13/58) of babies born at 39 weeks or later. However, in those that were positive for MMc, there was no correlation with the quantity of MMc and gestation (data not shown). Other studies have also found no statistical association between gestation, maternal age or parity and the incidence or quantity of MMc in CB (Berry, *et al* 2004, Scaradavou, *et al* 1996). Intriguingly, the levels of foetal Tregs also decrease during foetal development and it is hypothesised that during this time, when stimulated with alloantigen, the foetus transitions from being prone

to tolerance induction towards an effector function, in what is termed the layered immune system hypothesis (Mold*, et al* 2010). Tolerance towards NIMA is associated with Tregs specific for maternal alloantigens and this tolerance can be lost in the absence of sufficient MMc exposure (Dutta*, et al* 2009, Matsuoka*, et al* 2006, Molitor-Dart*, et al* 2007). This highlights the need for further studies into tolerance towards NIMA and the dynamics of MMc and Tregs during foetal development.

An alternative explanation to the active/passive transfer of maternal cells during pregnancy is that maternal cells were transferred during labour (for example, placental tearing) or during the CB collection and processing. However, no statistically significant associations between MMc and the method of delivery, induction status or year of birth (banking practices and centre experience has changed considerably over time) were identified; although these were only available for a subset of the cohort. Speculation about maternal contamination during the CB collection process could be gleaned from the observation that in this study, despite the use of a more sensitive detection method, 27% of CBUs tested positive for MMc compared to a previous report of 38% that used SSP for detection (Scaradavou*, et al* 1996). The lower than expected proportion of CBUs positive for MMc could be because clinical grade CBUs were tested. Studies that used research grade CBUs could have a higher incidence of maternal contamination during the CB collection process, with those CBUs more likely to not be suitable for banking and thus were instead available for research purposes.

The detection of HLA that matched the CB NIMA may also not be of maternal origin. Contamination in the laboratory may have occurred, particularly because these were historical DNA samples that would have been tested multiple times in the laboratory, giving numerous opportunities for contamination. Strict precautions were taken whilst I was testing the DNA samples, such as the use of filter tips but this does not rule out contamination at earlier time points. Furthermore, the NIMA HLA was also assumed to be of maternal origin but it is also feasible that this originated from an older sibling (Dierselhuis, *et al* 2014). MMc was not statistically associated with parity but this information was only available for a small subset of the cohort.

6.5.3   Limitations and further work

The HLA targets that were selected for q-PCR assays were common amongst the cohort studies and one or more targets were present in 95% (*n*= 166/175) mothers evaluated. However, only 96 (57%) of these CBUs could be tested for the presence of MMc because the maternal HLA was not the NIMA or it was not informative when compared to the CBU HLA. Several improvements could be made to the panel of primers available for microchimaerism detection and quantification. The HLA marker panel should be expanded to include additional specificities, especially at class II HLA loci. Primers specific for other HLA targets were available from colleagues in the Netherlands but were not selected due to low sensitivity. Redesign of some primers and further optimisation of reaction parameters may therefore be required.

The study population included is biased towards CBU-maternal pairs where the NIMA HLA was informative and it is possible that MMc levels in CBUs that were compatible to the mother and thus were excluded from experiments, differed to those reported. Non-HLA polymorphic markers could also be included, such as Insertion/Deletion (InDel) polymorphisms (Alizadeh, *et al* 2002). q-PCR assays that target three InDel polymorphisms, S-03, S-06 and S-11A have been optimised and validated (see **Appendix C**). However, these targets were not included for MMc analysis because 1) a screen for these polymorphisms showed low informativity, 2) an additional genotyping step was required prior to chimaerism analysis and 3) InDel q-PCR was not compatible with the HLA q-PCR conditions and thus the assays were not directly comparable.

If the q-PCR assays are to be introduced routinely in the H&I laboratory, other amendments could be made to improve workflow. Each of the HLA and HCK q-PCR assays were optimised to a similar amplification efficiency, which will permit the relative quantification of chimaerism by comparison of HLA and HCK C_q values, without the requirement of running standard curves on each PCR plate. Relative quantification is generally preferred over the absolute quantification (which uses the standard curve method) method used in this thesis because it offers a higher through-put. The inclusion of standard curves currently takes up half of the 96 well plate and only 8 samples of unknown chimaerism quantity can be run in triplicate per plate. The comparative C_q method could permit around 14 samples in triplicate per plate. Another advantage of optimising the HLA and HCK q-PCR assays to a similar amplification efficiency is the future possibility of multiplexing the reactions. This will control for well-well variations between HLA

and HCK amplification, further increase the number of samples per plate and also allow multiple informative markers to be tested simultaneously. Multiplexing the assay would however, require a change in chemistry from SYBR Green to TaqMan probes with multiple fluorophores, which can be more expensive but also offer enhanced specificity.

The development of digital droplet PCR technology has permitted more sensitive analyses of chimaerism (Eikmans*, et al* 2014). Using this method, a single DNA molecule is captured within water in oil droplet partitions and after thermal cycling to end-point PCR, each droplet is read to determine the proportion of droplets with positive amplification. This allows the absolute number of starting copies to be accurately determined and the use of TaqMan probes corresponding to two different alleles, the relative proportion of each allele in the sample can be determined. The use of digital droplet PCR also provides the significant advantage that quantification is not limited by amplification efficiencies because data capture occurs at end-point PCR and thus would be more feasible for a larger panel of informative polymorphic markers.

6.5.4 Conclusions

A q-PCR assay was developed for the detection and quantification of MMc in CB by the targeting of NIMA HLA polymorphisms. When this was used to quantify MMc in CB samples, 27% (*n*= 26/96) tested positive for MMc. The presence of MMc was not associated with the majority of maternal characteristics studied but appeared to be detected more frequently in the CB of babies born at 38 weeks or less. Given that Tregs are also present at higher quantities in the foetus during

earlier stages of gestation, further work should explore the role of Tregs in the development of MMc in humans, to supplement existing studies in the mouse. Further work should also expand on the observation that the presence of MMc seemed to require two-way compatibility between the mother and foetus. The q-PCR assay will also serve as a valuable tool that will enable a direct analysis of the influence of MMc in the CB graft on recipient clinical outcomes after transplantation and testing of the hypothesis that MMc mediates a GvL effect in recipients.

Chapter 7 General discussion and conclusions

7.1 Aims, major findings and further work

Donor-recipient HLA matching is crucial in the success of allogeneic unrelated HSCT (Eapen, *et al* 2011b, Lee, *et al* 2007, Petersdorf 2008) but the extensive polymorphism of HLA and their population-specific distribution influences the availability of a MUD, especially for non-Caucasian patients (Gragert, *et al* 2014). CB has been shown to extend the availability of a suitably HLA matched donor for ethnic minorities but TRM and aGvHD remain a challenge following HLA mismatched CBT (Barker, *et al* 2010a, Barker, *et al* 2010b, Eapen, *et al* 2014). Alternative HLA matching strategies: for example, the consideration of permissible HLA MMs such as NIMA, are therefore clinically needed. The central aim of this thesis was to explore the role of NIMA matching in unrelated CBT, with regards to donor availability and clinical outcome and to also develop a sensitive method to quantify the levels of MMc present in CBUs.

7.1.1   Generation of virtual phenotypes (chapter 3) and the provision of virtual full matches for patients (chapter 4)

The first aim of this thesis (chapter 3) was to define the *HLA-A*, *-B* and *-DRB1* NIMA of banked CBUs with maternal HLA types available, to generate VPs derived from the NIMA and to evaluate whether these increase the number and diversity of HLA phenotypes available for donor searches. A population genetics analysis of mothers and CBUs identified significant diversity in the representation of HLA alleles and haplotypes. Local algorithms were developed to identify the NIMA and to calculate the VPs derived from the 4,671 CBUs with maternal HLA types banked in the NHS-CBB. The actual number of phenotypes obtained from 4,671 CBUs increased 14.2-fold compared to the possible maximum 26-fold by the

generation of VPs due to incidences of non-informative NIMA. Maternal homozygosity and CBU-maternal 0 MM were generally comparable between HLA loci but differed at the population level. This meant that proportionally, a higher number of VPs were generated for BLK and MIX CBUs compared to CAU and ASI CBUs. A greater degree of HLA diversity was also present amongst BLK and MIX CBUs and maternal HLA typing CBUs from these groups may provide the greatest benefit to the size and diversity of the NHS-CBB, with minimal financial costs and also the greatest benefit to patients; especially given BLK patients are at a significant disadvantage with regards to donor availability. Knowledge of the probability of the NIMA being informative based on information from the CB HLA type could also aid the decision on whether to perform maternal HLA typing in patient specific cases. This could be particularly useful in situations whereby only a mismatched CB donor is available for a patient and the maternal HLA type is not available to check for a NIMA match. In these cases, it would also be beneficial if a "NIMA match probability" tool was available, where the mismatched patient allele is checked against the frequency of NIMA alleles within the donor ethnic group. The algorithms developed in the thesis and the analysis of NIMA allele and haplotype frequencies are the first steps in the development of such a tool.

The primary aim of chapter 4 was to determine whether the inclusion of VPs, to provide VFMs, can improve donor availability for patients of various ethnicities requiring CBT. Donor searches were performed on both the BBMR and BMDW registries for 457 patients. VFMs were available for patients without an IFM and thus when incorporated into the inherited match donor search strategy, VFMs, with adequate TNC dose, were able to extend the availability of a suitable donor

for patients requiring a transplant and in particular, for patients of ethnicities other than EC. VFMs from the BBMR doubled the cumulative availability of a matched donor for EC patients and tripled the availability for OE patients. VFMs from the BBMR also significantly increased donor availability for difficult to match patients with one or more infrequent HLA alleles. These findings provide evidence for the existence of VPs as true phenotypes in the patient population that were not yet present in the BBMR or BMDW registries.

The NHS-CBB aims to expand the number of banked CBUs to 50,000 but models suggest a 6/6 HLA match will be identified for only 9% of non-North West European patients based on an inventory of this size (Querol, *et al* 2009) and the introduction of more stringent HLA matching criteria is likely to further restrict availability. This thesis has shown that VPs provided a significant increase to the size of the donor pool and were able to provide VFMs for patients without an IFM. The generation of VPs and the prospective consideration of NIMA matches in CBT therefore seems to be a feasible alternative or complementary approach to increasing the number of banked CBUs in the NHS-CBB to improve donor availability.

There are current plans to retrospectively explore VFM donor availability for all sequential patients whereby a donor search was previously administered through Anthony Nolan over a four-year period (over 9,000 patients). This will capture patients that may not have proceeded to transplant due to the lack of a suitable donor and the large number of patients will permit further stratification of various ethnic groups. We are currently developing a tool that searches the virtual and inherited phenotypes of CB donors listed with the BBMR to identify

IFM and VFMs. This tool builds on the current study in that it estimates allelic resolution inherited and NIMA matches and *HLA-A*, *-B*, *-C* and *-DRB1*, from estimated second field HLA types, based on allele and haplotype frequencies. It also identifies mismatched donors that are only partially NIMA matched such as where one of two MMs is a NIMA match (6/8 + 1 NIMA). Furthermore, the automation of donor searches as opposed to manual searches using the BMDW Match Programs will enable the capture and analysis of NIMA matching in more detail, such as the likelihood of NIMA matching individual HLA alleles (such as *HLA-A*02:01*).

Continued periodic analyses of both inherited and virtual phenotypes of CB donors in the BBMR should be adopted. This will allow the monitoring of the diversity present amongst the VPs and whether these are providing additional diversity to the phenotypes present amongst the entire CB donor population in the BBMR. It is well established for inherited phenotypes that as the number of phenotypes increases, the proportion of these that are new phenotypes not yet represented decreases because only the less frequent phenotypes are not yet present. It is unclear whether this will be observed for VPs and if so, what the optimum virtual registry size will be. It is possible that once a certain threshold is reached, only VPs from ethnic groups underrepresented in the BBMR will provide a significant cost-benefit and the decision could be made to stop performing maternal HLA typing of Caucasian CB donors, especially since these were less likely to be informative for NIMA. These decisions are particularly important when there is a need to sustain the economic future of public CBBs

given the decreasing utilisation of CB donors (Magalon, *et al* 2015, Niederwieser, *et al* 2016, Passweg, *et al* 2016).

7.1.2   Association of NIMA matching with clinical outcomes (chapter 5)

The primary aim of chapter 5 was to retrospectively determine whether NIMA matching for *HLA-A*, *-B*, *-C* and *–DRB1* had any effect on clinical outcomes after single CBT in recipients with malignant and non-malignant disease. Unfortunately, the cohort was not of sufficient sample size to achieve an adequate statistical power able to detect differences in outcome between NIMA and non NIMA matched transplants. This highlights the need for a multi-centre international collaboration that considers NIMA matching in the context of four loci allelic resolution donor-recipient matching, especially given that I identified NIMA matches at *HLA-C*.

The effect of NIMA matching in the context of the updated recommended criteria of allelic resolution matching at *HLA-A*, *-B*, *-C* and *-DRB1* remains unknown. An observational retrospective study using registry data is in my opinion, the more feasible option to providing scientific evidence regarding the NIMA effect in CBT, compared the gold standard of a randomised, prospective clinical trial for several reasons. There is often a prohibitive financial cost associated with clinical trials and the niche field of NIMA matching will mean it is not a priority. Recruitment would likely be difficult and although half of patients had a VFM from BMDW, this would likely decrease once further HLA typing and other variables such as CBB are considered. Therefore at least half of patients will be excluded from

enrolment before other demographics such as disease indication are even considered.

A retrospective study offers the unique advantage that large numbers of heterogeneous patients can be followed for long periods of time, providing a more realistic estimate of the influence of NIMA matching and analyses of late complications after CBT, such as cGvHD. A Eurocord-CIBMTR collaboration combining data from the NYCB, NHS-CBB and other banks would provide a large cohort available for analyses. If more CBBs are recruited to provide maternal HLA types of transplanted CBUs, more specific research questions could be asked, such as the impact of patient disease indication (malignant versus non-malignant) or disease risk and whether there is any difference between NIMA matching at class I versus class II HLA loci or whether additional numbers of NIMA matches provide an additive effect.

The effect of NIMA matching in double CBT has also not yet been addressed and would be worthwhile given adults are unlikely to be considered for a single graft given the difficulty in achieving adequate cell dose. However, such a study will be difficult to conduct. Consideration of NIMA matching would require the maternal HLA types of both CBUs to be defined, which could be difficult given that the two units could have come from different CBBs that may differ in policy. Analyses of HLA matching in double CBT are already complex and it is unclear whether matching between the two units should be considered (Ballen and Lazarus 2016, Tees, *et al* 2016). Given that NIMA matching has been associated with improved engraftment in single CBT (van Rood, *et al* 2009), would NIMA matching in double

CBT affect unit dominance? Or could NIMA matching between the two CBUs lead to tolerance and the persistence of dual chimaerism?

7.1.3 Maternal microchimaerism in cord blood (chapter 6)

The primary aim of chapter 6 was to develop a sensitive q-PCR assay for the detection and quantification of MMc in CB, to determine the levels of MMc in transplanted CBUs and to investigate variables associated with its presence and/or absence. An absolute quantification method, that used standard curves were used to quantify the amount of NIMA HLA target in unknown samples, normalised against the total template DNA of the sample, quantified by an endogenous control, HCK. Initial q-PCR reactions of artificial spiking experiments were set up to test the protocol, optimise the conditions and characterise the assay performance by following the MIQE guidelines. The assays had a sensitivity of 0.01%.

This thesis detected MMc in 27% of 96 CB samples tested and found that MMc occurred more frequently in CB samples of babies born at 38 weeks or less. MMc is critical in the generation of NIMA-specific tolerance, likely through the generation of NIMA-specific Tregs (Dutta, *et al* 2009, Mold, *et al* 2008, Molitor-Dart, *et al* 2008). If MMc levels diminish with increasing gestation, it is possible the reduced level of exposure could negatively affect tolerance; especially given the foetus is proposed to transition towards an environment that favours an effector response during later gestational time points, which may not promote development of NIMA specific Tregs (Mold, *et al* 2008, Mold, *et al* 2010). Although clinical evidence in CBT is not available to support this, it may be that NIMA

matching in CBT is only beneficial to recipient outcomes in the context of MMc and indeed, NIMA matched donors have previously been selected on the basis of MMc in historic cases of haploidentical transplantation (Ichinohe, *et al* 2004). The selection of NIMA matched CB donors of babies born at earlier gestational time points may provide increased opportunity for the MMc to be present, although gestational information is not readily available during donor selection.

MMc was also significantly associated with compatibility at *HLA-A* but only when compatibility was from the perspective of both the mother and foetus, i.e. in both the GvH and HvG direction. This raises the possibility that there is a two-way interaction between maternal microchimaeric cells and the foetus that permits the passage and/or survival of MMc in the foetal host. Such bi-directional alloreactivity has been implicated as necessary for the tolerance of maternal renal grafts (Jankowska-Gan, *et al* 2012). Functional capacity of MMc in CB grafts has also been linked to a GvL response in recipients with one or more IPA matches to the CB in transplantation (van Rood, *et al* 2012). If additional clinical evidence becomes available to support this hypothesis, there may be disease specific cases where a CB donor positive for MMc is preferred over a donor with undetectable levels of MMc. Selection of CB donors born to babies of earlier gestational time, that are compatible to the mother in both the GvH and HvG direction and are matched to the patient IPA therefore may benefit patients with malignant disease.

Tolerance towards NIMA due to sufficient levels and/or quality of MMc appears, in the majority of cases, to be restricted to indirect pathways of allorecognition, without effect on the direct pathway, as discussed in the introduction (Akiyama, *et al* 2011, Bracamonte-Baran, *et al* 2017, Claas, *et al* 1988, Hadley, *et al* 1990,

Jankowska-Gan, *et al* 2012, Roelen, *et al* 1995, van Halteren, *et al* 2009). This split-tolerance towards NIMA is proposed to confer a reproductive advantage but is of significance in the transplantation setting (Bracamonte-Baran, *et al* 2017, Kinder, *et al* 2015). Female mice show an increased incidence in acute rejection of NIMA+ heart allografts compared to males in the absence of immunosuppression in mice (Molitor-Dart, *et al* 2008). Although no differences between male and female recipients of NIMA matched transplants in humans have been observed, NIMA matched grafts are associated with complications at early time points compared to NIPA and yet have a long term survival benefit in both renal and haploidentical transplantation (Burlingham, *et al* 1998, Kanda, *et al* 2009). Thus the hypothesised reproductive advantage conferred from a higher level of MMc in the development of Tregs may come at a significant cost in that it leads to stronger semi-direct pathway, responsible for higher acute rejection (Bracamonte-Baran, *et al* 2017, Kinder, *et al* 2015). The challenge to transplantation will be to manage this split-tolerance so that the natural MMc induced Treg mediated tolerance will benefit the recipient whilst the functional alloreactivity pathway is managed, possibly through targeted immunosuppression at specific time points.

A retrospective survival analysis of MMc and recipient outcomes after CBT could test for an association between the two and could also test the hypothesis that maternal cells sensitised against CB IPAs elicit a GvL response in the recipient (van Rood, *et al* 2012). The significant efforts of this research in the development of a central database containing maternal, CB and recipient demographics and clinical outcome provides an opportunity to perform such a retrospective

analysis. Adequate statistical power could be achieved if the panel of q-PCR microchimaerism markers is expanded and/or a collaboration with other CBBs are formed. An obvious example of a collaborative environment is the International Histocompatibility Working Group, which has fostered an international exchange of biological materials, clinical data and methodologies to advance our knowledge of histocompatibility and transplantation since the beginning of the field.

7.1.4 Haploidentical HSCT as an opportunity to study the relationship and immunological consequences of NIMA, MMc and Tregs

The recent decline in CBT and upwards trend in related haploidentical donor transplantation due to improved conditioning regimens, economic feasibility and donor accessibility and speed, warrant re-exploration of NIMA and donor choice in the modern era of haploidentical transplantation (Bertaina, *et al* 2017). Clinical trials and single centre studies could provide valuable information on the clinical outcomes of transplants using different types of haploidentical donors (parents, NIMA or NIPA matched sibling), either as a specific study or as a side arm. Non-registry studies would permit prospective analyses of more intricate concepts.

For example, immune regulation status of donor-recipient pairs, especially indirect pathway responses towards donor cell lysates containing all HLA and minor histocompatibility NIMAs, could be evaluated prior to transplant (Jankowska-Gan, *et al* 2012). The contribution of Tregs towards the regulation of alloreactivity against NIMA could also be measured by the recovery of effector functions and/or proliferation when Tregs are removed or added into the assay.

MMc levels could also be quantified and tested for an association with pre-transplant bi-directional alloreactivity. The results of these assays could be used to test for an association between pre-transplant immune regulation and clinical outcomes after HSCT. Ultimately, this could be used to provide insight into the prediction of whether tolerance or sensitisation towards NIMA is likely to occur in specific donor-recipient cases. Furthermore, co-infusing selected or expanded Tregs have recently been shown to prevent lethal aGvHD in haploidentical HSCT (Di Ianni, *et al* 2011) and this concept could be tested in cases of NIMA matched grafts and especially those where pre-transplant assessment predicted sensitisation, rather than tolerance, towards NIMA.

7.2 Additional applications of methodologies described in this thesis

There are several extensions to the methodologies and tools developed and their application in general to other clinical scenarios. Some are presented below, together with a description of how these have already been implemented or practical suggestions for their implementation.

7.2.1 HLA haplotype identification

The identification of CB donor HLA haplotypes has several clinical uses in transplantation. In adult donor HSCT, donor-recipient HLA haplotype matching has shown improved clinical outcomes compared to matching HLA phenotypes alone (Petersdorf, *et al* 2007). It is possible that additional MHC polymorphisms not considered in matching are allogeneic, given that MUD HSCT confers an increased risk of GvHD compared to HLA identical sibling donor HSCT (Yakoub-

Agha 2016). Furthermore, the CB IPA and IMA can also be distinguished by comparison of maternal and CBU HLA types. Early evidence suggests the anti-leukemic effect of CBT is due to the transfer of anti-IPA maternal immune cells in the CB graft to the recipient and IPA matching may be beneficial (van Rood, *et al* 2012). Knowledge of haplotype segregation will permit further analyses into the influence of donor-recipient IPA matching on recipient outcomes after CBT and the possibility of including IPA in the BMDW Match Programs.

Comparison of maternal and CB HLA types has also provided an important additional quality assurance step in the collection and banking of NHS-CBB CBUs. Although a rare occurrence, it is possible for the incorrect HLA type of CBUs to be reported and listed with registries, for reasons such as sample mix up during laboratory testing. The identity of the CBU is confirmed prior transplantation by the completion of confirmatory HLA typing on an additional blood segment attached to the CBU itself but the identification of a quality incident from identity confirmation could be many years after the CBU was processed and banked. However, if the mother's HLA type is defined during the processing and initial testing stage of banking, a comparison to the CBU HLA type can also be made during this time. The H&I laboratory at Colindale has now introduced an additional quality step to the process of HLA typing CB donors: each batch of CB HLA typing results are run through an algorithm (based on the one described in this thesis) to compare CB-maternal HLA types and identify any discrepancies, prior to the listing of donors with BBMR and BMDW.

Automated calculation of HLA haplotype segregation could also be used as a tool in family studies for related-donor transplantation. At present, identification of

HLA identical and haploidentical matched related donors is generally performed manually and automation of this analysis would increase efficiency. The NMDP has implemented an "HLA pedigree analysis tool" (www.pedigree.haplostats.org) where the HLA types of a patient and their relatives can be entered to identify matched donors. The tool could also be coupled with HLA haplotype frequencies to predict missing HLA typing at individual loci (such as *HLA-C)*. Comparison of allele strings between related individuals could also be used to eliminate non-shared alleles from ambiguous typing data and provide an HLA typing result with an improved resolution.

7.2.2 Retrospective analyses of clinical outcomes of recipients issued with a CBU from the NHS-CBB

The NHS-CBB holds the UKs largest observational studies of clinical information on CBT, with data available for over 500 issued CBUs over a twenty-year period that can be used for research purposes. At the start of this research, information that was not required for donor selection but could be relevant for research purposes, was not held in a central location and data was held across multiple electronic and paper sources. This restricted the ease of completing research studies using the available data.

During the course of this research, I have developed a controlled, standardised, central database that holds all relevant donor and recipient information. Clinical outcome data has been quality checked and manipulated for ease of future statistical analyses (e.g. grouping of similar disease indications together, calculation of time-to-event with the inclusion of censoring at follow-up and

competing events). HLA typing data from Eurocord and NHSBT has been combined to complete missing information and manipulated to a consistent, standardised format suitable for data mining. Allelic resolution HLA typing was also made available for a significant proportion of recipients and donors. Calculations performed during this research, such as donor-recipient HLA match grades have also been added. I have also collaborated with the statistics department, who will now look after the CBT data in much the same way as currently in place for solid organ transplantation. Looking forward, this means that future researchers will not need repeat very time-consuming processes required before they can even begin to address any research questions and the sharing of data between departments will hopefully improve, leading to more beneficial research that will ultimately improve patient care.

7.2.3   Post-transplant monitoring of chimaerism

Chimaerism monitoring plays an important role in post-transplant monitoring such as in the identification and monitoring of relapse, GvHD, graft failure and unit dominance in double CBT (Clark, *et al* 2015). The results can influence clinical decisions, such as whether to increase in immunosuppression or perform a donor lymphocyte infusion. The q-PCR assay for chimaerism detection and quantification could also be used for post-transplant monitoring. DNA extraction from different cell lineages would also allow chimaerism quantification of purified cell populations, such as B cells for monitoring disease relapse in a patient with a B cell lymphoma.   Chimaerism analyses for clinical purposes should report the coefficient of variance for a minimum of three informative markers (Clark, *et al* 2015) and thus a q-PCR assay that targeted polymorphisms

other than HLA (of which most loci are matched in HSCT), such as InDel polymorphisms, may be more suited for clinical use. A InDel specific q-PCR assay was optimised and validated as part of this thesis (see **Appendix C**) and the panel of markers could readily be expanded.

7.3 Conclusions and recommendations

Based on the findings of this thesis and the existing evidence in the literature, recommendations for maternal HLA typing of banked CBUs, selection of a NIMA matched donor and further analysis of clinical outcomes are given in **Table 7.1**.

This thesis has shown that performing maternal HLA tying of banked CBUs and identification of the CB NIMA led to a significant increase in the donor pool by the generation of VPs. These VPs were able to extend the availability of a suitable donor for patients requiring a transplant and in particular, for patients of ethnicities other than EC. This suggests the prospective provision of a NIMA matched CB donor is feasible, although there is a need for clinical evidence that supports NIMA matching in the modern era of allelic matching at *HLA-A*, *-B*, *-C* and *-DRB1* in CBT. This thesis has also contributed through the development of a sensitive q-PCR assay for MMc quantification and by the identification of gestation and maternal-foetal bi-directional alloreactivity as factors associated with the presence of MMc. Given that recent literature suggests that the tolerogenic response to NIMA is dependent on the level or type of MMc and that these microchimaeric cells may elicit a GvL response in CBT, an assay that quantifies MMc will be of use in the testing for an association with MMc and

clinical outcomes after CBT and in the future, MMc may even be considered during donor selection.

Table 7.1 Recommendations

1) Maternal HLA typing of NHS-CBB donors
a) The NHS-CBB should continue to perform maternal HLA typing
b) This should especially be performed for CBUs of Black and Mixed ethnicity
c) Typing should be performed at high resolution for *HLA-A*, *-B*, *-C* and *-DRB1*
2) UK cord blood donor selection
a) NIMA matching should be considered in the absence of a fully matched CB donor for patients with malignant disease
b) This should include careful weighting of other graft selection factors e.g. TNC dose, CBB accreditation status, ABO matching and DSA
c) The CB advisory committee must be included in any discussions of selecting a NIMA matched donor
d) The time taken to identify a suitable donor should not be extended for the purposes of trying to identify a NIMA match
3) NIMA matching and recipient clinical outcomes after cord blood transplantation
a) Studies of NIMA matching should be based on allelic resolution *HLA-A, -B, -C* and *-DRB1*
b) Malignant and non-malignant indications should be analysed separately
c) This should be an international, multi-centre collaboration, such as a joint Eurocord-CIBMTR analysis
4) Cord blood maternal microchimaerism and recipient clinical outcomes after transplantation
a) A standard method of chimaerism detection with comparable sensitivity between laboratories should be utilised
b) Donor-recipient shared IPA status should be taken into consideration
c) The effect of T cell depletion (e.g. use of ATG) should be included
d) This should be an international, multi-centre collaboration, such as the IHWG

References

Abbas, A.K., Lichtman, A.H. & Pillai, S. (2015) *Cellular and molecular immunology.* Elsevier/Saunders, Philadelphia, PA.

Akiyama, Y., Caucheteux, S.M., Vernochet, C., Iwamoto, Y., Tanaka, K., Kanellopoulos-Langevin, C. & Benichou, G. (2011) Transplantation tolerance to a single noninherited MHC class I maternal alloantigen studied in a TCR-transgenic mouse model. *J Immunol,* **186,** 1442-1449.

Alizadeh, M., Bernard, M., Danic, B., Dauriac, C., Birebent, B. & Lapart, C. (2002) Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. *Blood,* **99,** 4618-4625.

Andrassy, J., Kusaka, S., Jankowska-Gan, E., Torrealba, J.R., Haynes, L.D., Marthaler, B.R., Tam, R.C., Illigens, B.M., Anosova, N., Benichou, G. & Burlingham, W.J. (2003) Tolerance to noninherited maternal MHC antigens in mice. *J Immunol,* **171,** 5554-5561.

Apperley, J., Carreras, E., Gluckman, E. & Masszi, T. (2012) *Haematopoietic Stem Cell Transplantation-The EBMT Handbook.* European School Hematology.

Araki, M., Hirayama, M., Azuma, E., Kumamoto, T., Iwamoto, S., Toyoda, H., Ito, M., Amano, K. & Komada, Y. (2010) Prediction of reactivity to noninherited maternal antigen in MHC-mismatched, minor histocompatibility antigen-matched stem cell transplantation in a mouse model. *J Immunol,* **185,** 7739-7745.

Armitage, S., Warwick, R., Fehily, D., Navarrete, C. & Contreras, M. (1999) Cord blood banking in London: the first 1000 collections. *Bone Marrow Transplant,* **24,** 139-145.

Ballen, K.K. & Lazarus, H. (2016) Cord blood transplant for acute myeloid leukaemia. *Br J Haematol,* **173,** 25-36.

Barker, C.F. & Markmann, J.F. (2013) Historical overview of transplantation. *Cold Spring Harb Perspect Med,* **3,** a014977.

Barker, J.N., Byam, C.E., Kernan, N.A., Lee, S.S., Hawke, R.M., Doshi, K.A., Wells, D.S., Heller, G., Papadopoulos, E.B., Scaradavou, A., Young, J.W. & van den Brink, M.R.M. (2010a) Availability of Cord Blood Extends Allogeneic Hematopoietic Stem Cell Transplant Access to Racial and Ethnic Minorities. *Biology of Blood and Marrow Transplantation,* **16,** 1541-1548.

Barker, J.N., Scaradavou, A. & Stevens, C.E. (2010b) Combined effect of total nucleated cell dose and HLA match on transplantation outcome in 1061 cord blood recipients with hematologic malignancies. *Blood,* **115,** 1843-1849.

Benn, P. (2016) Expanding non-invasive prenatal testing beyond chromosomes 21, 18, 13, X and Y. *Clin Genet,* **90,** 477-485.

Berry, S.M., Hassan, S.S., Russell, E., Kukuruga, D., Land, S. & Kaplan, J. (2004) Association of maternal histocompatibility at class II HLA loci with maternal microchimerism in the fetus. *Pediatr Res,* **56,** 73-78.

Bertaina, A., Pitisci, A., Sinibaldi, M. & Algeri, M. (2017) T Cell-Depleted and T Cell-Replete HLA-Haploidentical Stem Cell Transplantation for Non-malignant Disorders. *Curr Hematol Malig Rep*.

Bettencourt, B.F., Santos, M.R., Fialho, R.N., Couto, A.R., Peixoto, M.J., Pinheiro, J.P., Spinola, H., Mora, M.G., Santos, C., Brehm, A. & Bruges-Armas, J. (2008) Evaluation of two methods for computational HLA haplotypes inference using a real dataset. *BMC Bioinformatics,* **9,** 68.

Billingham, R.E., Brent, L. & Medawar, P.B. (1953) Actively acquired tolerance of foreign cells. *Nature,* **172,** 603-606.

Billingham, R.E., Lampkin, G.H., Medawar, P.B. & Williams, H.L.L. (1952) Tolerance to homografts, twin diagnosis, and the freemartin condition in cattle. *Heredity,* **6,** 201-212.

Bracamonte-Baran, W. & Burlingham, W. (2015) Non-inherited maternal antigens, pregnancy, and allotolerance. *Biomed J,* **38,** 39-51.

Bracamonte-Baran, W., Florentin, J., Zhou, Y., Jankowska-Gan, E., Haynes, W.J., Zhong, W., Brennan, T.V., Dutta, P., Claas, F.H., van Rood, J.J. & Burlingham, W.J. (2017) Modification of host dendritic cells by microchimerism-derived extracellular vesicles generates split tolerance. *Proc Natl Acad Sci U S A*.

Brady, C., Paunic, V., Haagenson, M., Ruggeri, A., Gluckman, E., Rocha, V., Eapen, M., Maiers, M. & Spellman, S. (2015) LBP26: The NIMA effect in cord blood transplant: Real or a consequence of better high resolution matching in the NIMA matched group? *Human Immunology,* **76,** 231.

Brown, J.A. & Boussiotis, V.A. (2008) Umbilical cord blood transplantation: Basic biology and clinical challenges to immune reconstitution. *Clinical Immunology,* **127,** 286-297.

Brunstein, C., Eapen, M., Ahn, K.W., Appelbaum, F.R., Ballen, K.K., Champlin, R., Kan, F.Y., Laughlin, M.J., Soiffer, R.J., Weisdorf, D.J., Woolfrey, A., Horowitz, M.M. & Wagner, J.E. (2010) Reduced Intensity Conditioning (RIC) Transplantation In Acute Leukemia: The Effect of Source of Unrelated Donor Stem Cells on Outcomes. *Blood,* **116,** 400-401.

Buhler, S. & Sanchez-Mazas, A. (2011) HLA DNA sequence variation among human populations: molecular signatures of demographic and selective events. *PLoS One,* **6,** e14643.

Burlingham, W.J., Grailer, A.P., Fechner, J.H., Jr., Kusaka, S., Trucco, M., Kocova, M., Belzer, F.O. & Sollinger, H.W. (1995) Microchimerism linked to cytotoxic T lymphocyte functional unresponsiveness. *Transplantation,* **59,** 1147-1155.

Burlingham, W.J., Grailer, A.P., Heisey, D.M., Claas, F.H., Norman, D., Mohanakumar, T., Brennan, D.C., de Fijter, H., van Gelder, T., Pirsch, J.D., Sollinger, H.W. & Bean, M.A. (1998) The effect of tolerance to noninherited

maternal HLA antigens on the survival of renal transplants from sibling donors. *N Engl J Med,* **339,** 1657-1664.

Burt, T.D. (2013) Fetal regulatory T cells and peripheral immune tolerance in utero: implications for development and disease. *Am J Reprod Immunol,* **69,** 346-358.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. & Wittwer, C.T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem,* **55,** 611-622.

Campana, S., De Pasquale, C., Carrega, P., Ferlazzo, G. & Bonaccorsi, I. (2015) Cross-dressing: an alternative mechanism for antigen presentation. *Immunol Lett,* **168,** 349-354.

Cao, K., Moormann, A.M., Lyke, K.E., Masaberg, C., Sumba, O.P., Doumbo, O.K., Koech, D., Lancaster, A., Nelson, M., Meyer, D., Single, R., Hartzman, R.J., Plowe, C.V., Kazura, J., Mann, D.L., Sztein, M.B., Thomson, G. & Fernandez-Vina, M.A. (2004) Differentiation between African populations is evidenced by the diversity of alleles and haplotypes of HLA class I loci. *Tissue Antigens,* **63,** 293-325.

Carrington, M., Nelson, G.W., Martin, M.P., Kissner, T., Vlahov, D., Goedert, J.J., Kaslow, R., Buchbinder, S., Hoots, K. & O'Brien, S.J. (1999) HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science,* **283,** 1748-1752.

Castelli, E.C., Mendes-Junior, C.T., Veiga-Castelli, L.C., Pereira, N.F., Petzl-Erler, M.L. & Donadi, E.A. (2010) Evaluation of computational methods for the reconstruction of HLA haplotypes. *Tissue Antigens,* **76,** 459-466.

Chaix, R., Cao, C. & Donnelly, P. (2008) Is Mate Choice in Humans MHC-Dependent? *PLoS Genetics,* **4,** e1000184.

Chang, Y.J., Luznik, L., Fuchs, E.J. & Huang, X.J. (2016) How do we choose the best donor for T-cell-replete, HLA-haploidentical transplantation? *J Hematol Oncol,* **9,** 35.

Claas, F.H., Gijbels, Y., van der Velden-de Munck, J. & van Rood, J.J. (1988) Induction of B cell unresponsiveness to noninherited maternal HLA antigens during fetal life. *Science,* **241,** 1815-1817.

Clark, J.R., Scott, S.D., Jack, A.L., Lee, H., Mason, J., Carter, G.I., Pearce, L., Jackson, T., Clouston, H., Sproul, A., Keen, L., Molloy, K., Folarin, N., Whitby, L., Snowden, J.A., Reilly, J.T. & Barnett, D. (2015) Monitoring of chimerism following allogeneic haematopoietic stem cell transplantation (HSCT): technical recommendations for the use of short tandem repeat (STR) based techniques, on behalf of the United Kingdom National External Quality Assessment Service for Leucocyte Immunophenotyping Chimerism Working Group. *Br J Haematol,* **168,** 26-37.

Collett, D. (2015) *Modelling survival data in medical research.* CRC press.

Cupedo, T., Nagasawa, M., Weijer, K., Blom, B. & Spits, H. (2005) Development and activation of regulatory T cells in the human fetus. *Eur J Immunol,* **35,** 383-390.

Darrasse-Jèze, G., Darasse-Jèze, G., Klatzmann, D., Charlotte, F., Salomon, B.L. & Cohen, J.L. (2006) CD4+CD25+ regulatory/suppressor T cells prevent allogeneic fetus rejection in mice. *Immunol Lett,* **102,** 106-109.

Darrasse-Jèze, G., Marodon, G., Salomon, B.L., Catala, M. & Klatzmann, D. (2005) Ontogeny of CD4+CD25+ regulatory/suppressor T cells in human fetuses. *Blood,* **105,** 4715-4721.

Dausset, J. (1958) [Iso-leuko-antibodies]. *Acta Haematol,* **20,** 156-166.

Davey, S., Armitage, S., Rocha, V., Garnier, F., Brown, J., Brown, C.J., Warwick, R., Fehily, D., Watt, S., Gluckman, E., Vora, A., Contreras, M. & Navarrete, C.V.

(2004) The London Cord Blood Bank: analysis of banking and transplantation outcome. *Br J Haematol,* **125,** 358-365.

Di Ianni, M., Falzetti, F., Carotti, A., Terenzi, A., Castellino, F., Bonifacio, E., Del Papa, B., Zei, T., Ostini, R.I., Cecchini, D., Aloisi, T., Perruccio, K., Ruggeri, L., Balucani, C., Pierini, A., Sportoletti, P., Aristei, C., Falini, B., Reisner, Y., Velardi, A., Aversa, F. & Martelli, M.F. (2011) Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood,* **117,** 3921-3928.

Dierselhuis, M.P., Blokland, E.C., Pool, J., Schrama, E., Scherjon, S.A. & Goulmy, E. (2012) Transmaternal cell flow leads to antigen-experienced cord blood. *Blood,* **120,** 505-510.

Dierselhuis, M.P., Jankowska-Gan, E., Blokland, E., Pool, J., Burlingham, W.J., van Halteren, A.G. & Goulmy, E. (2014) HY immune tolerance is common in women without male offspring. *PLoS One,* **9,** e91274.

Drabbels, J.J., van de Keur, C., Kemps, B.M., Mulder, A., Scherjon, S.A., Claas, F.H. & Eikmans, M. (2011) HLA-targeted flow cytometric sorting of blood cells allows separation of pure and viable microchimeric cell populations. *Blood,* **118,** e149-155.

Dutta, P., Dart, M.L., Schumacher, S.M. & Burlingham, W.J. (2010) Fetal microchimerism persists at high levels in c-kit stem cells in sensitized mothers. *Chimerism,* **1,** 51-55.

Dutta, P., Molitor-Dart, M., Bobadilla, J.L., Roenneburg, D.A., Yan, Z., Torrealba, J.R. & Burlingham, W.J. (2009) Microchimerism is strongly correlated with tolerance to noninherited maternal antigens in mice. *Blood,* **114,** 3578-3587.

Eapen, M., Klein, J.P., Ruggeri, A., Spellman, S., Lee, S.J., Anasetti, C., Arcese, W., Barker, J.N., Baxter-Lowe, L.A., Brown, M., Fernandez-Vina, M.A., Freeman, J., He, W., Iori, A.P., Horowitz, M.M., Locatelli, F., Marino, S., Maiers, M., Michel, G., Sanz, G.F., Gluckman, E., Rocha, V. & Center for International

Blood and Marrow Transplant Research, N., E.rocord, and the European Group for Blood and Marrow Transplantation (2014) Impact of allele-level HLA matching on outcomes after myeloablative single unit umbilical cord blood transplantation for hematologic malignancy. *Blood,* **123,** 133-140.

Eapen, M., Klein, J.P., Sanz, G.F., Spellman, S., Ruggeri, A., Anasetti, C., Brown, M., Champlin, R.E., Garcia-Lopez, J., Hattersely, G., Koegler, G., Laughlin, M.J., Michel, G., Nabhan, S.K., Smith, F.O., Horowitz, M.M., Gluckman, E. & Rocha, V. (2011a) Effect of donor-recipient HLA matching at HLA A, B, C, and DRB1 on outcomes after umbilical-cord blood transplantation for leukaemia and myelodysplastic syndrome: a retrospective analysis. *Lancet Oncol,* **12,** 1214-1221.

Eapen, M., Le Rademacher, J., Antin, J.H., Champlin, R.E., Carreras, J., Fay, J., Passweg, J.R., Tolar, J., Horowitz, M.M., Marsh, J.C.W. & Deeg, H.J. (2011b) Effect of stem cell source on outcomes after unrelated donor transplantation in severe aplastic anemia. *Blood,* **118,** 2618-2621.

Eapen, M., Rocha, V., Sanz, G., Scaradavou, A., Zhang, M.J., Arcese, W., Sirvent, A., Champlin, R.E., Chao, N., Gee, A.P., Isola, L., Laughlin, M.J., Marks, D.I., Nabhan, S., Ruggeri, A., Soiffer, R., Horowitz, M.M., Gluckman, E., Wagner, J.E., Ctr Int Blood Marrow, T. & New York Blood, C. (2010) Effect of graft source on unrelated donor haemopoietic stem-cell transplantation in adults with acute leukaemia: a retrospective analysis. *Lancet Oncology,* **11,** 653-660.

Eapen, M., Rubinstein, P., Zhang, M.J., Camitta, B.M., Stevens, C., Cairo, M.S., Davies, S.M., Doyle, J.J., Kurtzberg, J., Pulsipher, M.A., Ortega, J.J., Scaradavou, A., Horowitz, M.M. & Wagner, J.E. (2006) Comparable long-term survival after unrelated and HLA-matched sibling donor. *J Clin Oncol,* **24,** 145-151.

Eapen, M., Rubinstein, P., Zhang, M.J., Stevens, C., Kurtzberg, J., Scaradavou, A., Loberiza, F.R., Champlin, R.E., Klein, J.P., Horowitz, M.M. & Wagner, J.E. (2007) Outcomes of transplantation of unrelated donor umbilical cord

blood and bone marrow in children with acute leukaemia: a comparison study. *Lancet,* **369,** 1947-1954.

Eikmans, M., van Halteren, A.G., van Besien, K., van Rood, J.J., Drabbels, J.J. & Claas, F.H. (2014) Naturally acquired microchimerism: implications for transplantation outcome and novel methodologies for detection. *Chimerism,* **5,** 24-39.

Erlebacher, A. (2013) Immunology of the maternal-fetal interface. *Annu Rev Immunol,* **31,** 387-411.

Ferrara, J.L., Levine, J.E., Reddy, P. & Holler, E. (2009) Graft-versus-host disease. *Lancet,* **373,** 1550-1561.

Flomenberg, N., Baxter-Lowe, L.A., Confer, D., Fernandez-Vina, M., Filipovich, A., Horowitz, M., Hurley, C., Kollman, C., Anasetti, C., Noreen, H., Begovich, A., Hildebrand, W., Petersdorf, E., Schmeckpeper, B., Setterholm, M., Trachtenberg, E., Williams, T., Yunis, E. & Weisdorf, D. (2004) Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. *Blood,* **104,** 1923-1930.

Furst, D., Muller, C., Vucinic, V., Bunjes, D., Herr, W., Gramatzki, M., Schwerdtfeger, R., Arnold, R., Einsele, H., Wulf, G., Pfreundschuh, M., Glass, B., Schrezenmeier, H., Schwarz, K. & Mytilineos, J. (2013) High-resolution HLA matching in hematopoietic stem cell transplantation: a retrospective collaborative analysis. *Blood,* **122,** 3220-3229.

Gammill, H.S., Adams Waldorf, K.M., Aydelotte, T.M., Lucas, J., Leisenring, W.M., Lambert, N.C. & Nelson, J.L. (2011) Pregnancy, microchimerism, and the maternal grandmother. *PLoS One,* **6,** e24101.

Gammill, H.S., Stephenson, M.D., Aydelotte, T.M. & Nelson, J.L. (2015) Microchimerism in women with recurrent miscarriage. *Chimerism***,** 1-3.

Gershon, R.K. & Kondo, K. (1970) Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology,* **18,** 723-737.

Gielis, E.M., Ledeganck, K.J., De Winter, B.Y., Del Favero, J., Bosmans, J.L., Claas, F.H., Abramowicz, D. & Eikmans, M. (2015) Cell-Free DNA: An Upcoming Biomarker in Transplantation. *Am J Transplant,* **15,** 2541-2551.

Gorer, P.A. (1938) The antigenic basis of tumour transplantation. *The Journal of Pathology and Bacteriology,* **47,** 231-252.

Gragert, L., Eapen, M., Williams, E., Freeman, J., Spellman, S., Baitty, R., Hartzman, R., Rizzo, J.D., Horowitz, M., Confer, D. & Maiers, M. (2014) HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *N Engl J Med,* **371,** 339-348.

Gragert, L., Madbouly, A., Freeman, J. & Maiers, M. (2013) Six-locus high resolution HLA haplotype frequencies derived from mixed-resolution DNA typing for the entire US donor registry. *Human Immunology,* **74,** 1313-1320.

Gratwohl, A., Stern, M., Brand, R., Apperley, J., Baldomero, H., de Witte, T., Dini, G., Rocha, V., Passweg, J., Sureda, A., Tichelli, A. & Niederwieser, D. (2009) Risk score for outcome after allogeneic hematopoietic stem cell transplantation: a retrospective analysis. *Cancer,* **115,** 4715-4726.

Hadley, G.A., Phelan, D., Duffy, B.F. & Mohanakumar, T. (1990) Lack of T-cell tolerance of noninherited maternal HLA antigens in normal humans. *Hum Immunol,* **28,** 373-381.

Hiby, S.E., Walker, J.J., O'Shaughnessy K, M., Redman, C.W., Carrington, M., Trowsdale, J. & Moffett, A. (2004) Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med,* **200,** 957-965.

Hilscher, C., Vahrson, W. & Dittmer, D.P. (2005) Faster quantitative real-time PCR protocols may lose sensitivity and show increased variability. *Nucleic Acids Res,* **33,** e182.

Hough, R., Danby, R., Russell, N., Marks, D., Veys, P., Shaw, B., Wynn, R., Vora, A., Mackinnon, S., Peggs, K.S., Crawley, C., Craddock, C., Pagliuca, A., Cook, G., Snowden, J.A., Clark, A., Marsh, J., Querol, S., Parkes, G., Braund, H. & Rocha, V. (2016) Recommendations for a standard UK approach to incorporating umbilical cord blood into clinical transplantation practice: an update on cord blood unit selection, donor selection algorithms and conditioning protocols. *Br J Haematol,* **172,** 360-370.

Ichinohe, T., Uchiyama, T., Shimazaki, C., Matsuo, K., Tamaki, S., Hino, M., Watanabe, A., Hamaguchi, M., Adachi, S., Gondo, H., Uoshima, N., Yoshihara, T., Hatanaka, K., Fujii, H., Kawa, K., Kawanishi, K., Oka, K., Kimura, H., Itoh, M., Inukai, T., Maruya, E., Saji, H., Kodera, Y. & Transplantation, J.C.S.G.f.N.-C.H.S.C. (2004) Feasibility of HLA-haploidentical hematopoietic stem cell transplantation between noninherited maternal antigen (NIMA)-mismatched family members linked with long-term fetomaternal microchimerism. *Blood,* **104,** 3821-3828.

Jagasia, M., Arora, M., Flowers, M.E., Chao, N.J., McCarthy, P.L., Cutler, C.S., Urbano-Ispizua, A., Pavletic, S.Z., Haagenson, M.D., Zhang, M.J., Antin, J.H., Bolwell, B.J., Bredeson, C., Cahn, J.Y., Cairo, M., Gale, R.P., Gupta, V., Lee, S.J., Litzow, M., Weisdorf, D.J., Horowitz, M.M. & Hahn, T. (2012) Risk factors for acute GVHD and survival after hematopoietic cell transplantation. *Blood,* **119,** 296-307.

Janeway, C.A., Travers, P., Walport, M. & Shlomchik, M.J. (2008) *Immunobiology: The Immune System in Health and Disease.* Garland Science, New York.

Jankowska-Gan, E., Sheka, A., Sollinger, H.W., Pirsch, J.D., Hofmann, R.M., Haynes, L.D., Armbrust, M.J., Mezrich, J.D. & Burlingham, W.J. (2012) Pretransplant immune regulation predicts allograft outcome: bidirectional regulation

correlates with excellent renal transplant function in living-related donor-recipient pairs. *Transplantation,* **93,** 283-290.

Joffre, O.P., Segura, E., Savina, A. & Amigorena, S. (2012) Cross-presentation by dendritic cells. *Nat Rev Immunol,* **12,** 557-569.

Kanda, J. (2013) Effect of HLA mismatch on acute graft-versus-host disease. *Int J Hematol,* **98,** 300-308.

Kanda, J., Ichinohe, T., Shimazaki, C., Hamaguchi, M., Watanabe, A., Ishida, H., Yoshihara, T., Morimoto, A., Uoshima, N., Adachi, S., Inukai, T., Sawada, A., Oka, K., Itoh, M., Hino, M., Maruya, E., Saji, H., Uchiyama, T. & Kodera, Y. (2009) Long-term survival after HLA-haploidentical SCT from noninherited maternal antigen-mismatched family donors: impact of chronic GVHD. *Bone Marrow Transplant,* **44,** 327-329.

Kawase, T., Matsuo, K., Kashiwase, K., Inoko, H., Saji, H., Ogawa, S., Kato, S., Sasazuki, T., Kodera, Y., Morishima, Y. & Program, J.M.D. (2009) HLA mismatch combinations associated with decreased risk of relapse: implications for the molecular mechanism. *Blood,* **113,** 2851-2858.

Kawase, T., Morishima, Y., Matsuo, K., Kashiwase, K., Inoko, H., Saji, H., Kato, S., Juji, T., Kodera, Y., Sasazuki, T. & Program, J.M.D. (2007) High-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease and implication for its molecular mechanism. *Blood,* **110,** 2235-2241.

Khanna, R., Silins, S.L., Weng, Z., Gatchell, D., Burrows, S.R. & Cooper, L. (1999) Cytotoxic T cell recognition of allelic variants of HLA B35 bound to an Epstein-Barr virus epitope: influence of peptide conformation and TCR-peptide interaction. *European Journal of Immunology,* **29,** 1587-1597.

Kinder, J.M., Jiang, T.T., Ertelt, J.M., Xin, L., Strong, B.S., Shaaban, A.F. & Way, S.S. (2015) Cross-Generational Reproductive Fitness Enforced by Microchimeric Maternal Cells. *Cell,* **162,** 505-515.

Knobloch, C., Goldmann, S.F. & Friedrich, W. (1991) Limited T cell receptor diversity of transplacentally acquired maternal T cells in severe combined immunodeficiency. *J Immunol,* **146,** 4157-4164.

Kogler, G., Enczmann, J., Rocha, V., Gluckman, E. & Wernet, P. (2005) High-resolution HLA typing by sequencing for HLA-A, -B, -C, -DR, -DQ in 122. *Bone Marrow Transplant,* **36,** 1033-1041.

Kollman, C., Maiers, M., Gragert, L., Müller, C., Setterholm, M., Oudshoorn, M. & Hurley, C.K. (2007) Estimation of HLA-A, -B, -DRB1 Haplotype Frequencies Using Mixed Resolution Data from a National Registry with Selective Retyping of Volunteers. *Human Immunology,* **68,** 950-958.

Krogsgaard, M. & Davis, M.M. (2005) How T cells 'see' antigen. *Nat Immunol,* **6,** 239-245.

Kubo, H., Ikeda-Moore, Y., Kikuchi, A., Miwa, K., Nokihara, K., Schönbach, C. & Takiguchi, M. (1998) Residue 116 determines the C-terminal anchor residue of HLA-B*3501 and -B*5101 binding peptides but does not explain the general affinity difference. *Immunogenetics,* **47,** 256-263.

Kurtzberg, J., Prasad, V.K., Carter, S.L., Wagner, J.E., Baxter-Lowe, L.A., Wall, D., Kapoor, N., Guinan, E.C., Feig, S.A., Wagner, E.L. & Kernan, N.A. (2008) Results of the Cord Blood Transplantation Study (COBLT): clinical outcomes of unrelated donor umbilical cord blood transplantation in pediatric patients with hematologic malignancies. *Blood,* **112,** 4318-4327.

Lam, T.H., Shen, M., Chia, J.M., Chan, S.H. & Ren, E.C. (2013) Population-specific recombination sites within the human MHC region. *Heredity,* **111,** 131-138.

Lambert, N.C., Erickson, T.D., Yan, Z., Pang, J.M., Guthrie, K.A., Furst, D.E. & Nelson, J.L. (2004) Quantification of maternal microchimerism by HLA-specific real-time polymerase chain reaction: studies of healthy women and women with scleroderma. *Arthritis Rheum,* **50,** 906-914.

Lee, S.J., Klein, J., Haagenson, M., Baxter-Lowe, L.A., Confer, D.L., Eapen, M., Fernandez-Vina, M., Flomenberg, N., Horowitz, M., Hurley, C.K., Noreen, H., Oudshoorn, M., Petersdorf, E., Setterholm, M., Spellman, S., Weisdorf, D., Williams, T.M. & Anasetti, C. (2007) High-resolution donor-recipient HLA matching contributes to the success of. *Blood,* **110,** 4576-4583.

Lim, W.H., McDonald, S.P., Coates, P.T., Chapman, J.R., Russ, G.R. & Wong, G. (2016) Maternal compared with paternal donor kidneys are associated with poorer graft outcomes after kidney transplantation. *Kidney Int,* **89,** 659-665.

Liu, Q., Rojas-Canales, D.M., Divito, S.J., Shufesky, W.J., Stolz, D.B., Erdos, G., Sullivan, M.L., Gibson, G.A., Watkins, S.C., Larregina, A.T. & Morelli, A.E. (2016) Donor dendritic cell-derived exosomes promote allograft-targeting immune response. *J Clin Invest,* **126,** 2805-2820.

Loubiere, L.S., Lambert, N.C., Flinn, L.J., Erickson, T.D., Yan, Z., Guthrie, K.A., Vickers, K.T. & Nelson, J.L. (2006) Maternal microchimerism in healthy adults in lymphocytes, monocyte/macrophages and NK cells. *Lab Invest,* **86,** 1185-1192.

Lown, R.N. & Shaw, B.E. (2013) Beating the odds: factors implicated in the speed and availability of unrelated haematopoietic cell donor provision. *Bone Marrow Transplantation,* **48,** 210-219.

Magalon, J., Maiers, M., Kurtzberg, J., Navarrete, C., Rubinstein, P., Brown, C., Schramm, C., Larghero, J., Katsahian, S., Chabannon, C., Picard, C., Platz, A., Schmidt, A. & Katz, G. (2015) Banking or Bankrupting: Strategies for Sustaining the Economic Future of Public Cord Blood Banks. *PLoS One,* **10,** e0143440.

Marino, J., Babiker-Mohamed, M.H., Crosby-Bertorini, P., Paster, J.T., LeGuern, C., Germana, S., Abdi, R., Uehara, M., Kim, J.I., Markmann, J.F., Tocco, G. & Benichou, G. (2016a) Donor exosomes rather than passenger leukocytes initiate alloreactive T cell responses after transplantation. *Sci Immunol,* **1**.

Marino, J., Paster, J. & Benichou, G. (2016b) Allorecognition by T Lymphocytes and Allograft Rejection. *Front Immunol,* **7,** 582.

Marsh, S., Parham, P. & Barber, L. (1999) *The HLA FactsBook.* Academic Press, London.

Matsuno, N., Wake, A., Uchida, N., Ishiwata, K., Araoka, H., Takagi, S., Tsuji, M., Yamamoto, H., Kato, D., Matsuhashi, Y., Seo, S., Masuoka, K., Miyakoshi, S., Makino, S., Yoneyama, A., Kanda, Y. & Taniguchi, S. (2009) Impact of HLA disparity in the graft-versus-host direction on engraftment in adult patients receiving reduced-intensity cord blood transplantation. *Blood,* **114,** 1689-1695.

Matsuoka, K., Ichinohe, T., Hashimoto, D., Asakura, S., Tanimoto, M. & Teshima, T. (2006) Fetal tolerance to maternal antigens improves the outcome of allogeneic bone marrow transplantation by a CD4+ CD25+ T-cell-dependent mechanism. *Blood,* **107,** 404-409.

Medawar, P.B. (1944) The behaviour and fate of skin autografts and skin homografts in rabbits: A report to the War Wounds Committee of the Medical Research Council. *Journal of Anatomy,* **78,** 176-199.

Merindol, N., Charrier, E., Duval, M. & Soudeyns, H. (2011) Complementary and contrasting roles of NK cells and T cells in pediatric umbilical cord blood transplantation. *Journal of Leukocyte Biology,* **90,** 49-60.

Michaëlsson, J., Mold, J.E., McCune, J.M. & Nixon, D.F. (2006) Regulation of T cell responses in the developing human fetus. *J Immunol,* **176,** 5741-5748.

Mold, J.E. & McCune, J.M. (2012) Immunological tolerance during fetal development: from mouse to man. *Adv Immunol,* **115,** 73-111.

Mold, J.E., Michaëlsson, J., Burt, T.D., Muench, M.O., Beckerman, K.P., Busch, M.P., Lee, T.H., Nixon, D.F. & McCune, J.M. (2008) Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. *Science,* **322,** 1562-1565.

Mold, J.E., Venkatasubrahmanyam, S., Burt, T.D., Michaelsson, J., Rivera, J.M., Galkina, S.A., Weinberg, K., Stoddart, C.A. & McCune, J.M. (2010) Fetal and Adult Hematopoietic Stem Cells Give Rise to Distinct T Cell Lineages in Humans. *Science,* **330,** 1695-1699.

Molitor-Dart, M.L., Andrassy, J., Haynes, L.D. & Burlingham, W.J. (2008) Tolerance induction or sensitization in mice exposed to noninherited maternal antigens (NIMA). *Am J Transplant,* **8,** 2307-2315.

Molitor-Dart, M.L., Andrassy, J., Kwun, J., Kayaoglu, H.A., Roenneburg, D.A., Haynes, L.D., Torrealba, J.R., Bobadilla, J.L., Sollinger, H.W., Knechtle, S.J. & Burlingham, W.J. (2007) Developmental exposure to noninherited maternal antigens induces CD4+ T regulatory cells: relevance to mechanism of heart allograft tolerance. *J Immunol,* **179,** 6749-6761.

Moretta, A., Locatelli, F., Mingrat, G., Rondini, G., Montagna, D., Comoli, P., Gandossini, S., Montini, E., Labirio, M. & Maccario, R. (1999) Characterisation of CTL directed towards non-inherited maternal alloantigens in human cord blood. *Bone Marrow Transplant,* **24,** 1161-1166.

Muller, S.M., Ege, M., Pottharst, A., Schulz, A.S., Schwarz, K. & Friedrich, W. (2001) Transplacentally acquired maternal T lymphocytes in severe combined immunodeficiency: a study of 121 patients. *Blood,* **98,** 1847-1851.

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

Niederwieser, D., Baldomero, H., Szer, J., Gratwohl, M., Aljurf, M., Atsuta, Y., Bouzas, L.F., Confer, D., Greinix, H., Horowitz, M., Iida, M., Lipton, J., Mohty, M., Novitzky, N., Nunez, J., Passweg, J., Pasquini, M.C., Kodera, Y., Apperley, J., Seber, A. & Gratwohl, A. (2016) Hematopoietic stem cell transplantation activity worldwide in 2012 and a SWOT analysis of the Worldwide Network for Blood and Marrow Transplantation Group including the global survey. *Bone Marrow Transplant,* **51,** 8.

Opelz, G. (1990) Analysis of the "NIMA effect" in renal transplantation. Collaborative Transplant Study. *Clin Transpl*, 63-67.

Owen, R.D. (1945) Immunogenetic consequences of vascular anastomoses between bovine twins. *Science,* **102,** 400-401.

Owen, R.D., Wood, H.R., Foord, A.G., Sturgeon, P. & Baldwin, L.G. (1954) Evidence for actively acquired tolerance to Rh antigens. *Proc Natl Acad Sci U S A,* **40,** 420-424.

Panajotopoulos, N., Ianhez, L.E., Neumann, J., Sabbaga, E. & Kalil, J. (1990) Immunological tolerance in human transplantation. The possible existence of a maternal effect. *Transplantation,* **50,** 443-445.

Parham, P. & Moffett, A. (2013) Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution. *Nat Rev Immunol,* **13,** 133-144.

Passweg, J.R., Baldomero, H., Bader, P., Bonini, C., Cesaro, S., Dreger, P., Duarte, R.F., Dufour, C., Kuball, J., Farge-Bancel, D., Gennery, A., Kroger, N., Lanza, F., Nagler, A., Sureda, A. & Mohty, M. (2016) Hematopoietic stem cell transplantation in Europe 2014: more than 40 000 transplants annually. *Bone Marrow Transplant,* **51,** 786-792.

Payne, R. (1957) Leukocyte agglutinins in human sera; correlation between blood transfusions and their development. *AMA Arch Intern Med,* **99,** 587-606.

Petersdorf, E.W. (2008) Optimal HLA matching in hematopoietic cell transplantation. *Curr Opin Immunol,* **20,** 588-593.

Petersdorf, E.W., Gooley, T.A., Malkki, M., Bacigalupo, A.P., Cesbron, A., Du Toit, E., Ehninger, G., Egeland, T., Fischer, G.F., Gervais, T., Haagenson, M.D., Horowitz, M.M., Hsu, K., Jindra, P., Madrigal, A., Oudshoorn, M., Ringden, O., Schroeder, M.L., Spellman, S.R., Tiercy, J.M., Velardi, A., Witt, C.S., O'Huigin, C., Apps, R. & Carrington, M. (2014) HLA-C expression levels define

permissible mismatches in hematopoietic cell transplantation. *Blood,* **124,** 3996-4003.

Petersdorf, E.W., Malkki, M., Gooley, T.A., Martin, P.J. & Guo, Z. (2007) MHC haplotype matching for unrelated hematopoietic cell transplantation. *PLoS Med,* **4,** e8.

Powley, L., Brown, C., Melis, A., Li, Y., Parkes, G. & Navarrete, C.V. (2016) Consideration of noninherited maternal Ags as permissible HLA mismatches in cord blood donor selection. *Bone Marrow Transplant,* **51,** 675-679.

Querol, S., Mufti, G.J., Marsh, S.G., Pagliuca, A., Little, A.M., Shaw, B.E., Jeffery, R., Garcia, J., Goldman, J.M. & Madrigal, J.A. (2009) Cord blood stem cells for hematopoietic stem cell transplantation in the UK: how big should the bank be? *Haematologica,* **94,** 536-541.

Rocha, V., Eapen, M., Scaradavou, A., Gluckman, E., Laughlin, M., Stevens, C., Horowitz, M.M., Wagner, J.E. & Ebmt (2009) Effect of stem cell source on transplant outcomes in adults with acute leukaemia: a comparison of unrelated bone marrow, peripheral blood and cord blood. *Bone Marrow Transplantation,* **43,** S5-S5.

Rocha, V. & Gluckman, E. (2009) Improving outcomes of cord blood transplantation: HLA matching, cell dose and other graft- and transplantation-related factors. *Br J Haematol,* **147,** 262-274.

Rocha, V., Spellman, S., Zhang, M.J., Ruggeri, A., Purtill, D., Brady, C., Baxter-Lowe, L.A., Baudoux, E., Bergamaschi, P., Chow, R., Freed, B., Koegler, G., Kurtzberg, J., Larghero, J., Lecchi, L., Nagler, A., Navarrette, C., Prasad, V., Pouthier, F., Price, T., Ratanatharathorn, V., van Rood, J.J., Horowitz, M.M., Gluckman, E. & Eapen, M. (2012) Effect of HLA-matching recipients to donor noninherited maternal antigens on outcomes after mismatched umbilical cord blood transplantation for hematologic malignancy. *Biol Blood Marrow Transplant,* **18,** 1890-1896.

Roelen, D.L., van Bree, F.P., van Beelen, E., van Rood, J.J. & Claas, F.H. (1995) No evidence of an influence of the noninherited maternal HLA antigens on the alloreactive T cell repertoire in healthy individuals. *Transplantation,* **59,** 1728-1733.

Rudolph, M.G., Stanfield, R.L. & Wilson, I.A. (2006) How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol,* **24,** 419-466.

Sakaguchi, S., Fukuma, K., Kuribayashi, K. & Masuda, T. (1985) Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J Exp Med,* **161,** 72-87.

Scaradavou, A., Carrier, C., Mollen, N., Stevens, C. & Rubinstein, P. (1996) Detection of maternal DNA in placental/umbilical cord blood by locus-specific amplification of the noninherited maternal HLA gene. *Blood,* **88,** 1494-1500.

Scrucca, L., Santucci, A. & Aversa, F. (2010) Regression modeling of competing risk using R: an in depth guide for clinicians. *Bone Marrow Transplant,* **45,** 1388-1395.

Shevach, E.M. (2011) Biological functions of regulatory T cells. *Adv Immunol,* **112,** 137-176.

Shiina, T., Hosomichi, K., Inoko, H. & Kulski, J.K. (2009) The HLA genomic loci map: expression, interaction, diversity and disease. *J Hum Genet,* **54,** 15-39.

Smits, J.M., Claas, F.H., van Houwelingen, H.C. & Persijn, G.G. (1998) Do noninherited maternal antigens (NIMA) enhance renal graft survival? *Transpl Int,* **11,** 82-88.

Snell, G.D. (1948) Methods for the study of histocompatibility genes. *J Genet,* **49,** 87-108.

Sommer, S. (2005) The importance of immune gene variability (MHC) in evolutionary ecology and conservation. *Front Zool,* **2,** 16.

Srivatsa, B., Srivatsa, S., Johnson, K.L. & Bianchi, D.W. (2003) Maternal cell microchimerism in newborn tissues. *The Journal of Pediatrics,* **142,** 31-35.

Stern, M., Ruggeri, L., Mancusi, A., Bernardo, M.E., de Angelis, C., Bucher, C., Locatelli, F., Aversa, F. & Velardi, A. (2008) Survival after T cell-depleted haploidentical stem cell transplantation is improved using the mother as donor. *Blood,* **112,** 2990-2995.

Takahashi, S., Ooi, J., Tomonari, A., Konuma, T., Tsukada, N., Oiwa-Monna, M., Fukuno, K., Uchiyama, M., Takasugi, K., Iseki, T., Tojo, A., Yamaguchi, T. & Asano, S. (2007) Comparative single-institute analysis of cord blood transplantation from unrelated donors with bone marrow or peripheral blood stem-cell transplants from related donors in adult patients with hematologic malignancies after myeloablative conditioning regimen. *Blood,* **109,** 1322-1330.

Tang, T.F., Hou, L., Chen, M., Belle, I., Mack, S., Lancaster, A., Ho, G.Y., Hwang, W.Y., Alsagoff, F., Ng, J. & Hurley, C.K. (2007) HLA haplotypes in Singapore: a study of mothers and their cord blood units. *Hum Immunol,* **68,** 430-438.

Tees, M.T., Betts, B., Hillgruber, R., Ayala, E., Field, T., Kharfan-Dabaja, M.A., Locke, F., Mishra, A., Nishihori, T., Ochoa-Bayona, J.L., Perez, L., Pidala, J., Anasetti, C., Fernandez, H. & Riches, M.L. (2016) New sources for argument: do HLA-C and HLA disparity in adult double umbilical cord blood transplants predict outcomes? *Bone Marrow Transplant,* **51,** 1256-1258.

Testi, M., Battarra, M., Lucarelli, G., Isgro, A., Morrone, A., Akinyanju, O., Wakama, T., Nunes, J.M., Andreani, M. & Sanchez-Mazas, A. (2015) HLA-A-B-C-DRB1-DQB1 phased haplotypes in 124 Nigerian families indicate extreme HLA diversity and low linkage disequilibrium in Central-West Africa. *Tissue Antigens,* **86,** 285-292.

Thompson, L.F., O'Connor, R.D. & Bastian, J.F. (1984) Phenotype and function of engrafted maternal T cells in patients with severe combined immunodeficiency. *J Immunol,* **133,** 2513-2517.

Tiercy, J.M. (2012) Unrelated hematopoietic stem cell donor matching probability and search algorithm. *Bone Marrow Res,* **2012,** 695018.

Trowsdale, J. (2011) The MHC, disease and selection. *Immunol Lett,* **137,** 1-8.

Tsafrir, A., Brautbar, C., Nagler, A., Elchalal, U., Miller, K. & Bishara, A. (2000) Alloreactivity of umbilical cord blood mononuclear cells: specific hyporesponse to noninherited maternal antigens. *Hum Immunol,* **61,** 548-554.

Tu, B., Leahy, N., Yang, R., Cha, N., Kariyawasam, K., Hou, L., Xiao, Y., Masaberg, C., Pulse-Earle, D., Maiers, M., Ng, J., Kurtzberg, J. & Hurley, C.K. (2013) Extensive haplotype diversity in African American mothers and their cord blood units. *Tissue Antigens,* **81,** 28-34.

van den Boogaardt, D.E., van Miert, P.P., Koekkoek, K.M., de Vaal, Y.J., van Rood, J.J., Claas, F.H. & Roelen, D.L. (2005) No in vitro evidence for a decreased alloreactivity toward noninherited maternal HLA antigens in healthy individuals. *Hum Immunol,* **66,** 1203-1212.

Van der Zanden, H.G., Van Rood, J.J., Oudshoorn, M., Bakker, J.N., Melis, A., Brand, A., Scaradavou, A. & Rubinstein, P. (2014) Noninherited maternal antigens identify acceptable HLA mismatches: benefit to patients and cost-effectiveness for cord blood banks. *Biol Blood Marrow Transplant,* **20,** 1791-1795.

van Halteren, A.G., Jankowska-Gan, E., Joosten, A., Blokland, E., Pool, J., Brand, A., Burlingham, W.J. & Goulmy, E. (2009) Naturally acquired tolerance and sensitization to minor histocompatibility antigens in healthy family members. *Blood,* **114,** 2263-2272.

van Rood, J.J. & Claas, F. (2000) Both self and non-inherited maternal HLA antigens influence the immune response. *Immunol Today,* **21,** 269-273.

Van Rood, J.J., Eernisse, J.G. & Van Leeuwen, A. (1958) Leucocyte antibodies in sera from pregnant women. *Nature,* **181,** 1735-1736.

van Rood, J.J., Loberiza, F.R., Zhang, M.J., Oudshoorn, M., Claas, F., Cairo, M.S., Champlin, R.E., Gale, R.P., Ringdén, O., Hows, J.M. & Horowitz, M.H. (2002) Effect of tolerance to noninherited maternal antigens on the occurrence of graft-versus-host disease after bone marrow transplantation from a parent or an HLA-haploidentical sibling. *Blood,* **99,** 1572-1577.

van Rood, J.J., Scaradavou, A. & Stevens, C.E. (2012) Indirect evidence that maternal microchimerism in cord blood mediates a graft-versus-leukemia effect in cord blood transplantation. *Proc Natl Acad Sci U S A,* **109,** 2509-2514.

van Rood, J.J., Stevens, C.E., Smits, J., Carrier, C., Carpenter, C. & Scaradavou, A. (2009) Reexposure of cord blood to noninherited maternal HLA antigens improves transplant outcome in hematological malignancies. *Proc Natl Acad Sci U S A,* **106,** 19952-19957.

Vernochet, C., Caucheteux, S.M., Gendron, M.C., Wantyghem, J. & Kanellopoulos-Langevin, C. (2005) Affinity-dependent alterations of mouse B cell development by noninherited maternal antigen. *Biol Reprod,* **72,** 460-469.

Wang, Y., Chang, Y.J., Xu, L.P., Liu, K.Y., Liu, D.H., Zhang, X.H., Chen, H., Han, W., Chen, Y.H., Wang, F.R., Wang, J.Z., Chen, Y., Yan, C.H., Huo, M.R., Li, D. & Huang, X.J. (2014) Who is the best donor for a related HLA haplotype-mismatched transplant? *Blood,* **124,** 843-850.

Wedekind, C., Seebeck, T., Bettens, F. & Paepke, A.J. (1995) MHC-dependent mate preferences in humans. *Proc Biol Sci,* **260,** 245-249.

Winternitz, J., Abbate, J.L., Huchard, E., Havlicek, J. & Garamszegi, L.Z. (2016) Patterns of MHC-dependent mate selection in humans and nonhuman primates: a meta-analysis. *Mol Ecol*, **26**, 668-688.

Woolfrey, A., Klein, J.P., Haagenson, M., Spellman, S., Petersdorf, E., Oudshoorn, M., Gajewski, J., Hale, G.A., Horan, J., Battiwalla, M., Marino, S.R., Setterholm, M., Ringden, O., Hurley, C., Flomenberg, N., Anasetti, C., Fernandez-Vina, M. & Lee, S.J. (2011) HLA-C Antigen Mismatch Is Associated with Worse Outcome in Unrelated Donor Peripheral Blood Stem Cell Transplantation. *Biology of blood and marrow transplantation,* **17,** 885-892.

Yakoub-Agha, I. (2016) Transplantations from HLA-identical siblings versus 10/10 HLA-matched unrelated donors. *Semin Hematol,* **53,** 74-76.

Zhang, L. & Miller, R.G. (1993) The correlation of prolonged survival of maternal skin grafts with the presence of naturally transferred maternal T cells. *Transplantation,* **56,** 918-921.

Appendix A – Chapter 3 supplementary material

Supplementary Table A.I CBU and maternal *HLA-A* allele frequencies. Frequencies were calculated by a direct count from 4,671 CBUs and their mothers and are given as percentages. *Total also includes frequencies in OE and UNK groups.

	CAU (*n*= 2,831)		ASI (*n*= 790)		SEA (*n*= 47)		BLK (*n*= 256)		MIX (*n*= 605)		Total* (*n*= 4,671)	
	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother
A*01	16.32	16.83	12.15	12.53	14.75	15.16	1.06	2.13	8.59	8.40	14.21	14.71
A*02	29.94	28.17	16.08	14.37	26.27	24.74	26.60	30.85	19.34	18.16	24.96	24.30
A*03	13.07	13.58	6.39	5.95	11.07	11.22	3.19	2.13	10.35	9.38	9.17	9.17
A*11	5.76	5.99	15.32	15.19	7.58	7.78	24.47	17.02	1.56	2.73	7.19	7.52
A*23	1.61	1.75	0.82	0.76	2.24	2.23	-	-	10.35	10.74	3.64	2.89
A*24	8.55	8.53	16.84	17.15	10.13	10.50	25.53	24.47	3.32	3.13	10.00	12.40
A*25	1.96	2.21	0.13	0.06	1.39	1.53	1.06	-	-	-	1.16	1.24
A*26	3.11	3.25	4.68	4.81	3.23	3.43	1.06	2.13	0.78	1.56	3.14	3.55
A*29	4.12	4.42	1.52	1.08	3.35	3.48	1.06	1.06	2.54	3.71	3.06	2.56
A*30	2.07	2.42	2.15	2.09	3.06	2.93	1.06	1.06	13.87	11.13	4.55	3.31
A*31	2.98	2.51	2.41	2.22	2.61	2.30	2.13	3.19	0.78	0.78	1.74	1.82
A*32	4.12	4.10	3.23	3.61	3.66	3.70	1.06	2.13	1.17	1.17	3.47	3.31
A*33	1.29	1.27	10.25	11.27	3.46	3.73	11.70	12.77	5.47	4.69	3.22	3.97
A*34	0.09	0.09	0.51	0.95	0.45	0.50	-	-	2.93	2.54	0.99	0.99
A*36	0.02	0.02	-	-	0.30	0.26	-	-	2.93	3.32	0.91	0.50
A*43	-	-	-	-	-	0.02	-	-	-	-	-	0.08
A*66	0.44	0.34	0.06	-	0.55	0.43	-	-	1.95	2.54	0.99	0.58
A*68	4.24	4.33	7.22	7.78	5.25	5.50	-	-	10.16	10.35	6.12	6.36
A*69	0.18	0.18	0.06	0.06	0.14	0.14	-	-	0.20	0.20	0.08	0.08
A*74	0.16	0.04	0.13	0.06	0.44	0.39	-	-	2.93	4.88	1.24	0.66
A*80	-	-	0.06	0.06	0.09	0.04	-	-	0.78	0.59	0.17	-

Supplementary Table A.II CBU and maternal *HLA-B* allele frequencies. Frequencies were calculated by a direct count from 4,671 CBUs and their mothers and are given as percentages. *Total also includes frequencies in OE and UNK groups.

	CAU (*n*= 2,831)		ASI (*n*= 790)		SEA (*n*= 47)		BLK (*n*= 256)		MIX (*n*= 605)		Total* (*n*= 4,671)	
	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother
B*07	13.92	14.38	5.76	5.25	11.37	11.58	2.13	3.19	9.38	8.79	9.42	9.50
B*08	10.54	11.27	5.57	4.56	8.93	9.27	-	-	3.52	4.88	8.76	8.68
B*13	2.23	2.24	2.85	2.91	2.31	2.39	7.45	3.19	1.17	0.98	2.40	3.06
B*14	3.78	3.92	0.70	0.57	2.90	3.01	-	1.06	2.34	2.34	2.48	2.48
B*15	6.91	6.38	9.68	9.49	8.08	7.77	17.02	12.77	12.50	13.67	8.18	8.18
B*18	5.16	4.91	2.85	3.29	4.30	4.30	-	-	2.15	2.73	3.64	4.21
B*27	4.12	3.85	1.58	1.71	3.39	3.29	4.26	3.19	1.37	1.17	3.47	3.97
B*35	8.05	7.82	13.04	13.35	9.33	9.06	6.38	7.45	8.40	5.86	10.58	10.17
B*37	1.36	1.43	3.29	2.97	1.67	1.71	3.19	3.19	0.59	0.59	1.40	1.74
B*38	2.15	2.38	1.52	1.77	1.94	2.10	1.06	2.13	0.39	0.39	1.90	2.07
B*39	1.78	1.96	0.44	0.63	1.58	1.67	3.19	1.06	2.15	1.76	1.57	1.65
B*40	6.43	6.43	13.73	12.91	7.60	7.54	11.70	12.77	1.95	0.98	7.02	7.60
B*41	0.76	0.87	0.63	0.57	0.91	0.95	-	-	1.37	2.34	1.65	1.32
B*42	0.09	0.05	-	-	0.46	0.39	-	-	4.69	4.69	0.99	0.66
B*44	15.70	15.95	6.27	7.66	12.86	13.17	4.26	5.32	7.42	6.45	11.07	11.24
B*45	0.53	0.44	0.19	0.13	0.72	0.71	-	-	2.73	3.52	1.32	1.57
B*46	0.04	0.04	0.25	0.13	0.19	0.22	6.38	9.57	-	-	0.33	0.50
B*47	0.34	0.37	0.06	0.13	0.28	0.28	-	-	0.78	0.39	0.08	-
B*48	0.05	0.04	0.57	0.51	0.16	0.14	2.13	3.19	-	-	-	-
B*49	1.50	1.11	0.51	0.63	1.41	1.17	-	-	1.95	2.73	1.82	1.40
B*50	0.90	0.85	1.71	1.46	1.13	1.04	-	-	0.78	0.98	1.82	1.32
B*51	4.84	4.31	9.24	7.78	5.90	5.32	10.64	10.64	6.05	5.66	5.95	5.54
B*52	1.04	1.06	7.28	8.29	2.48	2.56	5.32	7.45	2.34	1.76	2.73	2.23

	CAU (*n*= 2,831)		ASI (*n*= 790)		SEA (*n*= 47)		BLK (*n*= 256)		MIX (*n*= 605)		Total* (*n*= 4,671)	
	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother
*B*53*	0.44	0.42	0.19	-	1.21	0.98	-	-	10.35	9.18	2.56	1.49
*B*54*	0.02	0.04	0.00	0.06	0.05	0.09	1.06	2.13	-	-	0.25	0.25
*B*55*	1.77	1.87	1.96	2.22	1.69	1.97	4.26	5.32	0.20	0.39	1.57	2.56
*B*56*	0.62	0.49	0.38	0.44	0.62	0.52	1.06	1.06	-	-	0.91	0.83
*B*57*	4.08	4.10	5.19	5.89	4.28	4.39	2.13	-	5.86	6.05	3.88	3.80
*B*58*	0.83	0.99	4.43	4.62	2.02	2.25	5.32	4.26	7.62	9.77	1.98	1.74
*B*67*	-	-	0.06	0.06	0.02	0.03	1.06	1.06	-	-	-	0.08
*B*73*	0.02	0.02	0.06	-	0.06	0.05	-	-	0.59	0.59	-	0.08
*B*78*	0.02	-	-	-	0.02	-	-	-	-	-	0.08	-
*B*81*	-	0.02	-	-	0.07	0.07	-	-	0.98	0.98	0.17	0.08
*B*82*	-	-	-	-	0.02	0.02	-	-	0.39	0.39	-	-

Supplementary Table A.III CBU and maternal *HLA-DRB1* allele frequencies. Frequencies were calculated by a direct count from 4,671 CBUs and their mothers and are given as percentages. *Total also includes frequencies in OE and UNK groups.

	CAU (*n*= 2,831)		ASI (*n*= 790)		SEA (*n*= 47)		BLK (*n*= 256)		MIX (*n*= 605)		Total* (*n*= 4,671)	
	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother	CBU	Mother
DRB1*01	11.76	10.88	3.73	3.61	9.61	9.05	3.19	4.26	6.45	8.01	9.09	8.60
DRB1*03	11.82	12.56	8.92	8.42	11.38	11.70	2.13	1.06	13.09	13.67	12.31	11.57
DRB1*04	18.28	17.15	9.75	10.19	15.42	14.78	13.83	9.57	6.25	5.08	12.81	14.21
DRB1*07	13.33	14.54	13.42	14.87	12.79	14.01	7.45	10.64	8.79	9.57	12.23	12.98
DRB1*08	2.35	2.74	1.08	1.52	2.69	2.99	3.19	4.26	8.59	8.59	3.64	3.55
DRB1*09	1.15	1.18	1.39	1.33	1.42	1.49	10.64	13.83	1.95	1.95	1.82	1.98
DRB1*10	0.83	0.71	5.38	5.38	1.91	1.93	3.19	3.19	2.73	4.10	1.90	2.07
DRB1*11	8.92	8.28	9.49	10.70	9.63	9.13	8.51	7.45	10.35	10.74	12.31	9.75
DRB1*12	1.93	2.19	4.62	4.87	2.57	2.93	14.89	15.96	2.15	2.93	1.82	2.98
DRB1*13	10.12	10.67	8.29	8.99	10.60	10.75	7.45	4.26	20.51	17.97	12.07	11.16
DRB1*14	2.70	2.79	8.48	7.66	3.84	3.77	8.51	4.26	0.98	1.37	4.21	4.30
DRB1*15	14.62	14.43	24.56	21.46	16.28	15.77	13.83	19.15	16.41	14.45	14.38	15.29
DRB1*16	2.19	1.89	0.89	1.01	1.85	1.71	3.19	2.13	1.76	1.56	1.40	1.57

Supplementary Figure A.I Cumulative frequency of CBU IMA/IPA and NIMA HLA A~B~DRB1 haplotypes according to broad population. Haplotypes were identified by comparing maternal and CBU HLA types in an identical-by-descent algorithm. The CBU IMA/IPA and NIMA haplotypes were combined to form a single dataset. Frequencies were calculated by a direct count and ranked in descending order. *Total also includes OE and UNK.
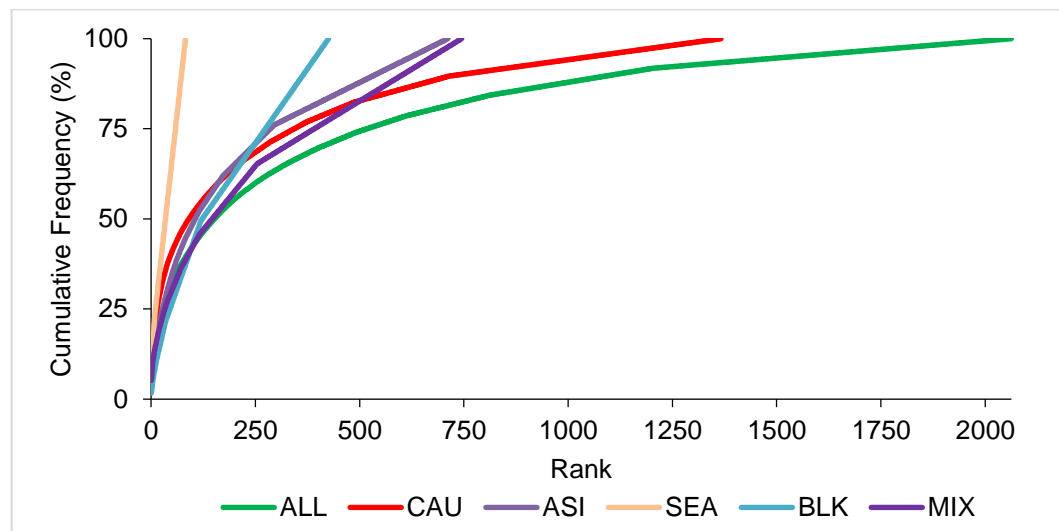
Supplementary Table A.IV Average cumulative CBU IMA/IPA and NIMA HLA A~B~DRB1 haplotype frequencies in each broad population. Haplotypes were identified by comparing maternal and CBU HLA types in an identical-by-descent algorithm. The CBU inherited and NIMA haplotypes were combined to form a single dataset. Frequencies are given as percentages and were calculated by a direct count and ranked in descending order. *Total also includes OE and UNK.

	Population					Total*
	CAU	**ASI**	**SEA**	**BLK**	**MIX**	
Rank						
10	21.88	13.79	23.96	8.58	14.15	16.40
25	31.04	23.22	39.58	16.50	21.59	23.92
50	40.75	34.60	65.62	26.40	30.08	32.24
100	51.52	48.56	-	42.90	42.25	42.47
250	68.45	70.80	-	70.96	64.61	59.89
500	82.77	-	-	-		74.31
Average						
Mean	0.07	0.14	1.20	0.23	0.14	0.05
Median	0.03	0.06	1.04	0.17	0.07	0.02

Supplementary Table A.V Frequency (F) of the top 10 ranking CBU IMA/IPA and NIMA HLA A~B~DRB1 haplotypes in each population and comparison to other NHS-CBB and NMDP populations. Haplotypes were identified by comparing maternal and CBU HLA types in an identical-by-descent algorithm. The CBU inherited and NIMA haplotypes were combined to form a single dataset. Frequencies are given as percentages and ranked in descending order. Frequencies in the NHS-CBB were calculated by a direct count. Second field frequencies in the NMDP were estimated by a modified version of the expectation-maximisation algorithm (Gragert, *et al* 2013), downloaded from http://frequency.nmdp.org/NMDPFrequencies2011/. These were collapsed back to first field and frequencies were re-calculated. Comparisons were made between the NHS-CBB CAU and the NMDP CAU populations, between the NHS-CBB ASI and NMDP Asia and Pacific Islander populations and between the NHS-CBB BLK and NMDP African American populations.

| | NHS-CBB CBU inherited and NIMA population | | | | | | | | | | NMDP | |
| | CAU | | ASI | | SEA | | BLK | | MIX | | | |
	Rank	F (%)	Rank	F (%)	Rank	F (%)	Rank	F (%)	Rank	F (%)	Rank	F (%)
CAU												
A*01~B*08~DRB1*03	**1**	**6.59**	19	0.57	-	-	14	0.50	1	5.17	1	6.01
A*03~B*07~DRB1*15	**2**	**2.93**	71	0.29	-	-	24	0.50	3	1.20	2	3.06
A*02~B*44~DRB1*04	**3**	**2.80**	-	-	-	-	7	0.66	2	1.91	3	2.17
A*29~B*44~DRB1*07	**4**	**2.13**	558	0.06	-	-	-	-	6	0.92	6	1.45
A*02~B*07~DRB1*15	**5**	**1.91**	43	0.40	-	-	15	0.50	5	0.99	4	1.94
A*01~B*57~DRB1*07	**6**	**1.37**	2	1.84	-	-	40	0.33	4	1.13	8	1.07
A*02~B*15~DRB1*04	**7**	**1.37**	185	0.11	-	-	151	0.17	12	0.57	5	1.47
A*03~B*35~DRB1*01	**8**	**1.07**	27	0.52	-	-	67	0.33	26	0.42	7	1.25
A*02~B*08~DRB1*03	**9**	**0.92**	33	0.46	-	-	-	-	22	0.42	10	0.79
A*02~B*57~DRB1*07	**10**	**0.78**	198	0.11	-	-	58	0.33	154	0.14	14	0.68
ASI												
A*33~B*44~DRB1*07	696	0.03	**1**	**2.87**	6	2.08	-	-	15	0.57	2	1.91
A*01~B*57~DRB1*07	6	1.37	**2**	**1.84**	-	-	40	0.33	4	1.13	5	1.46
A*26~B*08~DRB1*03	180	0.11	**3**	**1.55**	-	-	265	0.17	106	0.21	25	0.58
A*02~B*40~DRB1*15	56	0.25	**4**	**1.49**	-	-	49	0.33	18	0.50	9	0.90

| | NHS-CBB CBU inherited and NIMA population | | | | | | | | | | NMDP | |
| | CAU | | ASI | | SEA | | BLK | | MIX | | | |
	Rank	F (%)	Rank	F (%)	Rank	F (%)	Rank	F (%)	Rank	F (%)	Rank	F (%)
A*01~B*37~DRB1*10	114	0.14	**5**	**1.21**	35	1.04	-	-	10	0.57	16	0.79
A*11~B*52~DRB1*15	173	0.11	**6**	**1.21**	-	-	214	0.17	426	0.07	39	0.40
A*24~B*15~DRB1*12	978	0.02	**7**	**1.03**	58	1.04	-	-	65	0.28	10	0.88
A*33~B*58~DRB1*03	478	0.05	**8**	**1.03**	80	1.04	341	0.17	235	0.14	1	2.13
A*24~B*15~DRB1*15	427	0.05	**9**	**0.80**	59	1.04	-	-	472	0.07	20	0.72
A*01~B*40~DRB1*15	-	-	**10**	**0.75**	-	-	-	-	-	-	75	0.24
SEA												
A*02~B*46~DRB1*09	-	-	127	0.17	**1**	**5.21**	-	-	348	0.07	3	1.68
A*11~B*15~DRB1*04	205	0.10	28	0.52	**2**	**3.13**	-	-	403	0.07	17	0.78
A*02~B*40~DRB1*11	83	0.19	51	0.34	**3**	**2.08**	-	-	336	0.07	52	0.33
A*24~B*35~DRB1*15	342	0.06	23	0.57	**4**	**2.08**	259	0.17	99	0.21	37	0.42
A*24~B*48~DRB1*12	-	-	149	0.17	**5**	**2.08**	-	-	-	-	265	0.08
A*33~B*44~DRB1*07	696	0.03	1	2.87	**6**	**2.08**	-	-	15	0.57	2	1.91
A*02~B*40~DRB1*09	391	0.05	-	-	**7**	**2.08**	-	-	-	-	23	0.60
A*11~B*15~DRB1*14	-	-	137	0.17	**8**	**2.08**	-	-	-	-	130	0.15
A*33~B*13~DRB1*15	-	-	-	-	**9**	**2.08**	-	-	-	-	527	0.04
A*01~B*52~DRB1*15	78	0.19	84	0.23	**10**	**1.04**	-	-	43	0.28	172	0.12
BLK												
A*30~B*42~DRB1*03	-	-	-	-	-	-	**1**	**1.65**	39	0.35	1	1.73
A*34~B*44~DRB1*15	-	-	-	-	-	-	**2**	**0.99**	111	0.21	8	0.61
A*01~B*07~DRB1*03	-	-	-	-	-	-	**3**	**0.83**	257	0.07	1399	0.01
A*30~B*57~DRB1*13	-	-	-	-	-	-	**4**	**0.83**	600	0.07	17	0.46
A*66~B*58~DRB1*15	-	-	-	-	-	-	**5**	**0.83**	692	0.07	19	0.41
A*68~B*15~DRB1*13	705	0.03	679	0.06	-	-	**6**	**0.83**	-	-	48	0.24
A*02~B*44~DRB1*04	3	2.80	-	-	-	-	**7**	**0.66**	2	1.91	10	0.54

	NHS-CBB CBU inherited and NIMA population										NMDP	
	CAU		ASI		SEA		BLK		MIX			
	Rank	F (%)	Rank	F (%)	Rank	F (%)	Rank	F (%)	Rank	F (%)	Rank	F (%)
A*02~B*51~DRB1*08	105	0.16	-	-	-	-	**8**	**0.66**	150	0.14	371	0.06
A*23~B*15~DRB1*03	947	0.02	-	-	-	-	**9**	**0.66**	438	0.07	82	0.20
A*23~B*44~DRB1*07	15	0.65	476	0.06	-	-	**10**	**0.66**	9	0.71	104	0.18
MIX												
A*01~B*08~DRB1*03	1	6.59	19	0.57	-	-	14	0.50	**1**	**5.17**	-	-
A*02~B*44~DRB1*04	3	2.80	-	-	-	-	7	0.66	**2**	**1.91**	-	-
A*03~B*07~DRB1*15	2	2.93	71	0.29	-	-	24	0.50	**3**	**1.20**	-	-
A*01~B*57~DRB1*07	6	1.37	2	1.84	-	-	40	0.33	**4**	**1.13**	-	-
A*02~B*07~DRB1*15	5	1.91	43	0.40	-	-	15	0.50	**5**	**0.99**	-	-
A*29~B*44~DRB1*07	4	2.13	558	0.06	-	-	-	-	**6**	**0.92**	-	-
A*02~B*44~DRB1*07	12	0.73	70	0.29	14	3.13	-	-	**7**	**0.78**	-	-
A*30~B*13~DRB1*07	18	0.59	12	0.75	74	-	290	0.17	**8**	**0.78**	-	-
A*23~B*44~DRB1*07	15	0.65	476	0.06	-	-	10	0.66	**9**	**0.71**	-	-
A*01~B*37~DRB1*10	114	0.14	5	1.21	35	-	-	-	**10**	**0.57**	-	-

Supplementary Table A.VI NHS-CBB CBU IMA/IPA and NIMA HLA A~B~DRB1 haplotypes ranking in the bottom 25th centile of the corresponding estimated frequencies in the NMDP. Haplotypes were identified by comparing maternal and CBU HLA types in an identical-by-descent algorithm. The CBU inherited and NIMA HLA haplotypes were combined to form a single dataset. Frequencies are given as percentages and ranked in descending order. Frequencies in the NHS-CBB were calculated by a direct count. Second field frequencies in the NMDP were estimated by a modified version of the expectation-maximisation algorithm (Gragert, *et al* 2013), downloaded from http://frequency.nmdp.org/NMDPFrequencies2011/. These were collapsed back to first field and frequencies were re-calculated. The 75th centile covered the top 75% of the population when ranked in descending order of haplotype frequency.

	NHS-CBB		NMDP	
	F (%)	Rank	F (%)	Rank
CAU				
Below NMDP 75th centile				
A*29~B*37~DRB1*03	0.01593	1106	0.00009	4783
A*33~B*55~DRB1*03	0.01593	1285	0.00009	4866
A*33~B*78~DRB1*01	0.01593	1287	0.00001	6110
A*36~B*35~DRB1*15	0.01593	1290	0.00007	4979
A*68~B*48~DRB1*12	0.01593	1337	0.00003	5608
A*74~B*53~DRB1*16	0.01593	1365	0.00007	4963
Not seen in NMDP Caucasian				
A*30~B*81~DRB1*13	0.01593	1179	-	-
ASI				
Below NMDP 75th centile				
A*01~B*18~DRB1*08	0.05747	304	0.00031	3493
A*23~B*49~DRB1*10	0.05747	479	0.0002	3772
A*29~B*39~DRB1*13	0.05747	557	0.00007	4523
A*30~B*08~DRB1*10	0.05747	566	0.00028	3561
A*31~B*57~DRB1*12	0.05747	595	0.00032	3480
A*66~B*51~DRB1*11	0.05747	670	0.00017	3887
Not seen in NMDP Asia and Pacific				
A*01~B*47~DRB1*15	0.05747	321	-	-
A*01~B*73~DRB1*14	0.05747	340	-	-
A*03~B*08~DRB1*12	0.05747	399	-	-
A*23~B*14~DRB1*11	0.05747	475	-	-
A*25~B*27~DRB1*13	0.05747	524	-	-
A*25~B*40~DRB1*13	0.05747	525	-	-
A*74~B*45~DRB1*10	0.05747	711	-	-
SEA				
Below NMDP 75th centile				
A*31~B*14~DRB1*07	1.04167	28	0.00029	3542
Not seen in NMDP Asia and Pacific				
A*25~B*55~DRB1*14	1.04167	72	-	-
BLK				
Below NMDP 75th centile				

	NHS-CBB		NMDP	
	F (%)	**Rank**	**F (%)**	**Rank**
A*01~B*73~DRB1*08	0.33003	41	0.00037	4754
A*26~B*57~DRB1*09	0.16502	270	0.00012	5495
A*30~B*41~DRB1*10	0.16502	300	0.00031	4879
A*31~B*07~DRB1*08	0.16502	314	0.00031	4888
A*68~B*81~DRB1*04	0.16502	400	0.0001	5644
A*74~B*51~DRB1*14	0.16502	415	0.00009	5741
Not seen in NMDP African American				
A*03~B*47~DRB1*08	0.16502	198	-	-
A*03~B*73~DRB1*10	0.16502	209	-	-
A*26~B*38~DRB1*10	0.16502	268	-	-
A*69~B*51~DRB1*11	0.16502	402	-	-
Not seen in any NMDP population				
A*23~B*73~DRB1*07	0.16502	244	-	-

Supplementary Table A.VII Heterozygous CBU-Maternal 0 MM for the top 10 ranking *x, y* allele combinations at *HLA-A*, *-B* and –*DRB1*. Frequencies of individual heterozygous *x, y* allele combinations were calculated by a direct count. 0 MM are when the CBU and mother have identical first field *x, y* alleles at an HLA locus. Results show the number of 0 MM out of the total *x, y* alleles in each population. Total also includes OTH and UNK categories.

Rank	Total		CAU		ASI		SEA		BLK		MIX	
	x, y	0 MM	x, y	0 MM	x, y	0 MM	x, y	0 MM	x, y	0 MM	x, y	0 MM
HLA-A												
1	*01, *02	79/392	*01, *02	63/297	*02, *24	6/40	*02, *11	3/10	*02, *30	2/12	*01, *02	11/46
2	*02, *03	58/275	*02, *03	48/219	*11, *24	7/38	*11, *24	1/5	*02, *03	2/11	*02, *24	6/31
3	*02, *24	39/229	*02, *24	24/140	*02, *11	12/34	*02, *24	1/3	*02, *23	2/10	*02, *68	7/23
4	*02, *11	37/184	*02, *11	17/111	*11, *33	5/32	*02, *33	1/3	*01, *02	2/9	*02, *30	3/22
5	*01, *03	18/153	*01, *03	13/106	*01, *02	2/31	*24, *33	1/3	*02, *68	2/9	*01, *03	4/21
6	*02, *68	24/135	*02, *32	13/84	*01, *11	5/30	*11, *33	0/2	*23, *30	1/9	*02, *03	3/21
7	*01, *24	15/126	*02, *68	13/82	*24, *33	2/27	*01, *33	0/1	*03, *23	0/9	*01, *11	6/20
8	*03, *24	14/111	*01, *24	9/74	*01, *24	3/26	*11, *32	0/1	*01, *03	0/8	*01, *24	2/19
9	*02, *32	17/108	*02, *29	8/70	*02, *33	4/26	*02, *25	0/1	*01, *68	0/8	*02, *11	3/18
10	*01, *11	15/107	*03, *24	10/67	*03, *24	1/26	*02, *03	0/1	*02, *33	2/7	*02, *23	2/13
HLA-B												
1	*07, *44	15/139	*07, *44	11/116	*35, *51	6/26	*15, *27	0/3	*15, *35	0/7	*08, *44	2/13
2	*08, *44	24/133	*08, *44	21/106	*15, *40	4/25	*13, *51	0/2	*15, *44	0/7	*35, *40	2/12
3	*07, *08	15/106	*07, *08	13/91	*35, *40	3/22	*13, *52	0/2	*15, *53	0/7	*40, *44	1/12
4	*15, *44	5/100	*15, *44	5/70	*35, *52	3/20	*15, *35	0/2	*35, *53	0/6	*07, *35	1/12
5	*40, *44	10/88	*40, *44	8/63	*15, *51	3/18	*15, *39	0/2	*07, *58	2/6	*07, *44	2/12
6	*35, *44	9/85	*35, *44	7/62	*40, *52	2/18	*13, *35	0/1	*15, *57	0/5	*35, *51	0/11
7	*08, *35	7/83	*07, *15	4/55	*15, *35	3/17	*13, *46	0/1	*35, *58	0/5	*15, *35	0/10

Rank	Total		CAU		ASI		SEA		BLK		MIX	
	x, y	0 MM	x, y	0 MM	x, y	0 MM	x, y	0 MM	x, y	0 MM	x, y	0 MM
8	*07, *35	8/79	*07, *40	8/53	*15, *57	0/14	*13, *58	0/1	*44, *53	0/5	*15, *51	2/10
9	*07, *15	5/74	*08, *35	4/52	*08, *35	2/14	*15, *44	0/1	*51, *58	0/5	*08, *15	0/10
10	*15, *35	5/73	*18, *44	6/47	*40, *51	1/13	*15, *46	0/1	*07, *15	1/5	*15, *44	0/9
HLA-DRB1												
1	*04, *15	33/218	*04, *15	20/145	*07, *15	13/54	*09, *11	1/3	*03, *13	4/18	*13, *15	1/24
2	*07, *15	34/197	*04, *07	22/120	*04, *15	8/41	*09, *14	0/3	*13, *15	3/13	*03, *11	2/22
3	*04, *07	27/172	*01, *04	15/119	*14, *15	6/39	*11, *15	0/2	*03, *15	1/13	*11, *15	2/21
4	*03, *15	26/161	*03, *04	23/117	*11, *15	3/31	*12, *15	0/2	*11, *13	1/12	*04, *07	2/21
5	*03, *04	26/153	*07, *15	19/112	*03, *15	6/24	*12, *16	1/2	*04, *13	0/11	*03, *15	3/19
6	*03, *07	25/148	*03, *07	15/106	*13, *15	3/23	*14, *15	0/2	*07, *15	1/11	*03, *07	1/19
7	*04, *13	16/144	*03, *15	16/102	*10, *15	1/21	*03, *13	0/2	*11, *15	3/10	*04, *11	1/18
8	*01, *04	16/141	*04, *13	11/101	*12, *15	6/20	*07, *12	1/2	*08, *13	0/10	*07, *15	1/18
9	*13, *15	15/136	*01, *07	13/99	*03, *07	9/20	*01, *13	0/1	*01, *13	1/7	*01, *13	2/17
10	*01, *15	15/131	*01, *15	11/89	*04, *07	0/20	*01, *15	0/1	*01, *15	0/7	*03, *13	0/17

Appendix B – Chapter 4 supplementary material

Table B.I Virtual 6/6 matched CB donor availability according to patient ethnicity.

Patient Ethnicity	CB donor search strategy	
	BBMR	**BMDW**
European Caucasoid	86/274 (31.4%)	136/274 (50.0%)
Other ethnicity	17/67 (25.4%)	30/67 (44.8%)
• *Non-European Caucasoid*	10/35	15/35
• *Black*	2/13	6/13
• *Southeast Asian*	1/6	2/6
• *Mixed*	4/13	7/13
Unknown/ Not Declared	27/116 (23.3%)	55/116 (47.4%)
All Ethnicities	130/457 (28.4%)	221/457 (48.4%)

Table B.II Patient _HLA-A_, _-B_ and _-DRB1_ allele frequencies. Allele frequencies were calculated by a direct count method. Patient _n_= 457.

HLA-A						HLA-B						HLA-DRB1		
Allele	_F_ (%)	Cumulative _F_ (%)	Allele	_F_ (%)	Cumulative _F_ (%)	Allele	_F_ (%)	Cumulative _F_ (%)	Allele	_F_ (%)	Cumulative _F_ (%)	Allele	_F_ (%)	Cumulative _F_ (%)
A*02	24.7	24.7	B*44	15.0	15.0	B*50	1.1	93.7	DRB1*04	17.9	17.9
A*01	16.1	40.8	B*07	10.6	25.6	B*56	1.0	94.6	DRB1*15	12.8	30.7
A*03	10.8	51.6	B*15	8.1	33.7	B*41	0.9	95.5	DRB1*07	12.0	42.8
A*24	10.2	61.8	B*08	7.9	41.6	B*45	0.9	96.4	DRB1*13	11.6	54.4
A*11	8.1	69.9	B*35	7.9	49.5	B*53	0.9	97.3	DRB1*03	10.7	65.1
A*68	5.5	75.4	B*51	6.9	56.3	B*42	0.7	97.9	DRB1*11	9.3	74.4
A*26	4.6	80.0	B*40	5.9	62.3	B*47	0.5	98.5	DRB1*01	8.1	82.5
A*33	4.0	84.0	B*14	3.6	65.9	B*48	0.4	98.9	DRB1*14	4.5	87.0
A*29	3.3	87.3	B*27	3.6	69.5	B*60	0.2	99.1	DRB1*08	3.5	90.5
A*32	2.7	90.0	B*57	3.3	72.8	B*62	0.2	99.3	DRB1*16	3.1	93.5
A*30	2.5	92.6	B*18	3.1	75.8	B*73	0.2	99.6	DRB1*12	3.0	96.5
A*31	2.4	95.0	B*38	2.6	78.4	B*46	0.1	99.7	DRB1*09	1.9	98.4
A*23	2.2	97.2	B*52	2.6	81.1	B*54	0.1	99.8	DRB1*10	1.6	100.0
A*25	1.2	98.4	B*58	2.4	83.5	B*67	0.1	99.9			
A*34	0.5	98.9	B*49	2.3	85.8	B*81	0.1	100.0			
A*36	0.3	99.2	B*39	2.2	88.0						
A*66	0.3	99.6	B*13	1.8	89.7						
A*74	0.3	99.9	B*55	1.5	91.2						
A*69	0.1	100.0	B*37	1.3	92.6						

Table B.III Availability of a virtual 6/6 matched CB donor from BMDW according to patient *HLA-A, -B* and *-DRB1* alleles.

	BMDW virtual 6/6		
	Yes	**No**	**Total**
HLA-A			
*A*01*	82 (55.8)	65 (44.2)	147
*A*02*	136 (60.2)	90 (39.8)	226
*A*03*	57 (57.6)	42 (42.4)	99
*A*11*	27 (36.5)	47 (63.5)	74
*A*23*	7 (35)	13 (65)	20
*A*24*	47 (50.5)	46 (49.5)	93
*A*25*	3 (27.3)	8 (72.7)	11
*A*26*	14 (33.3)	28 (66.7)	42
*A*29*	12 (40)	18 (60)	30
*A*30*	7 (30.4)	16 (69.6)	23
*A*31*	10 (45.5)	12 (54.5)	22
*A*32*	5 (20)	20 (80)	25
*A*33*	13 (35.1)	24 (64.9)	37
*A*34*	1 (20)	4 (80)	5
*A*36*	0 (0)	3 (100)	3
*A*66*	0 (0)	3 (100)	3
*A*68*	20 (40)	30 (60)	50
*A*69*	0 (0)	1 (100)	1
*A*74*	1 (33.3)	2 (66.7)	3
HLA-B			
*B*07*	72 (74.2)	25 (25.8)	97
*B*08*	43 (59.7)	29 (40.3)	72
*B*13*	8 (50)	8 (50)	16
*B*14*	16 (48.5)	17 (51.5)	33
*B*15*	36 (48.6)	38 (51.4)	74
*B*18*	12 (42.9)	16 (57.1)	28
*B*27*	11 (33.3)	22 (66.7)	33
*B*35*	36 (50)	36 (50)	72
*B*37*	4 (33.3)	8 (66.7)	12
*B*38*	6 (25)	18 (75)	24
*B*39*	7 (35)	13 (65)	20
*B*40*	30 (55.6)	24 (44.4)	54
*B*41*	2 (25)	6 (75)	8
*B*42*	1 (16.7)	5 (83.3)	6
*B*44*	80 (58.4)	57 (41.6)	137

B*45	3 (37.5)	5 (62.5)	8
B*46	0 (0)	1 (100)	1
B*47	0 (0)	5 (100)	5
B*48	1 (25)	3 (75)	4
B*49	7 (33.3)	14 (66.7)	21
B*50	4 (40)	6 (60)	10
B*51	28 (44.4)	35 (55.6)	63
B*52	6 (25)	18 (75)	24
B*53	2 (25)	6 (75)	8
B*54	0 (0)	1 (100)	1
B*55	4 (28.6)	10 (71.4)	14
B*56	2 (22.2)	7 (77.8)	9
B*57	15 (50)	15 (50)	30
B*58	4 (18.2)	18 (81.8)	22
B*60	1 (50)	1 (50)	2
B*62	1 (50)	1 (50)	2
B*67	0 (0)	1 (100)	1
B*73	0 (0)	2 (100)	2
B*81	0 (0)	1 (100)	1
HLA-DRB1			
DRB1*01	34 (45.9)	40 (54.1)	74
DRB1*03	51 (52)	47 (48)	98
DRB1*04	102 (62.2)	62 (37.8)	164
DRB1*07	57 (51.8)	53 (48.2)	110
DRB1*08	5 (15.6)	27 (84.4)	32
DRB1*09	1 (5.9)	16 (94.1)	17
DRB1*10	2 (13.3)	13 (86.7)	15
DRB1*11	42 (49.4)	43 (50.6)	85
DRB1*12	6 (22.2)	21 (77.8)	27
DRB1*13	50 (47.2)	56 (52.8)	106
DRB1*14	15 (36.6)	26 (63.4)	41
DRB1*15	69 (59)	48 (41)	117
DRB1*16	9 (32.1)	19 (67.9)	28

Appendix C – Chapter 6 supplementary material

Table C.I InDel marker panel characteristics. InDel targets were chosen from a list of published polymorphic markers previously used in q-PCR assays for the detection of chimaerism. S-03, S-06 and S-11A were chosen because they were reported to be the most informative in a previously tested population (Alizadeh, *et al* 2002)**.**

Target	Chromosome location		Primer sequence (3' – 5')	Amplicon length (bp)	Mean amplicon T_m (°C)
S-03	6q	F	CTTTTGCTTTCTGTTTCTTAAGGGC	223	85.38 ± 0.20
		R	TCAATCTTTGGGCAGGTTGAA		
S-06	1p	F	CAGTCACCCCGTGAAGTCCT	106	84.24 ± 0.23
		R	TTTCCCCCATCTGCCTATTG		
S-11A	11	F	TAGGATTCAACCCTGGAAGC	189	81.10 ± 0.31
		R	CCAGCATGCACCTGACTAACA		

Table C.II InDel q-PCR thermal cycling parameters trialled with iQ-SYBR Green Supermix. InDel specific q-PCR reactions were not compatible with the SYBR Select MasterMix (Life technologies) used for the HLA specific assays, possibly due to a non-optimal $MgCl_2$ concentration in the master mix. InDel q-PCR reactions were set up using the iQ-SYBR Green Supermix (Bio-Rad) and by following the Netherlands recommended thermal cycling protocol for controls positive and negative for S-03, S-06 and S-11A. The Netherlands InDel protocol again did not show exponential amplification for any target (data not shown) and it was clear that the InDel thermal cycling protocol required further optimisation. The recommended thermal cycling protocol for the iQ-SYBR Green Supermix (Bio-Rad) was next trialled, which has a shorter initial denaturation step (5 minutes) compared to the Netherlands InDel thermal cycling protocol.

Parameter	Netherlands protocol		Bio-Rad protocol		Final protocol	
	Temp. (^{0}C)	Time	Temp. (^{0}C)	Time	Temp. (^{0}C)	Time
Initial denaturation	95	10:00	95	5:00	95	5:00
Cycling (x40)						
Denaturation	95	0:45	95	0:15	95	0:15
Annealing	65	1:00	55 – 65*	1:00	63	1:00

Figure C.I InDel q-PCR primer annealing temperature gradient dissociation curves. Primer annealing temperatures: **(A)** 56ºC, **(B)** 58ºC, **(C)** 60ºC, **(D)** 62ºC, **(E)** 64ºC and **(F)** 66ºC. Q-PCR reactions were set up using male platelet donor DNA positive for the target of interest and using a variable primer annealing temperature. Amplicons were continuously heated from 55^0C - 95^0C and temperature (^{0}C) was plotted against the derivative Reporter (-Rn') to determine the amplicon melting temperature (T_m).
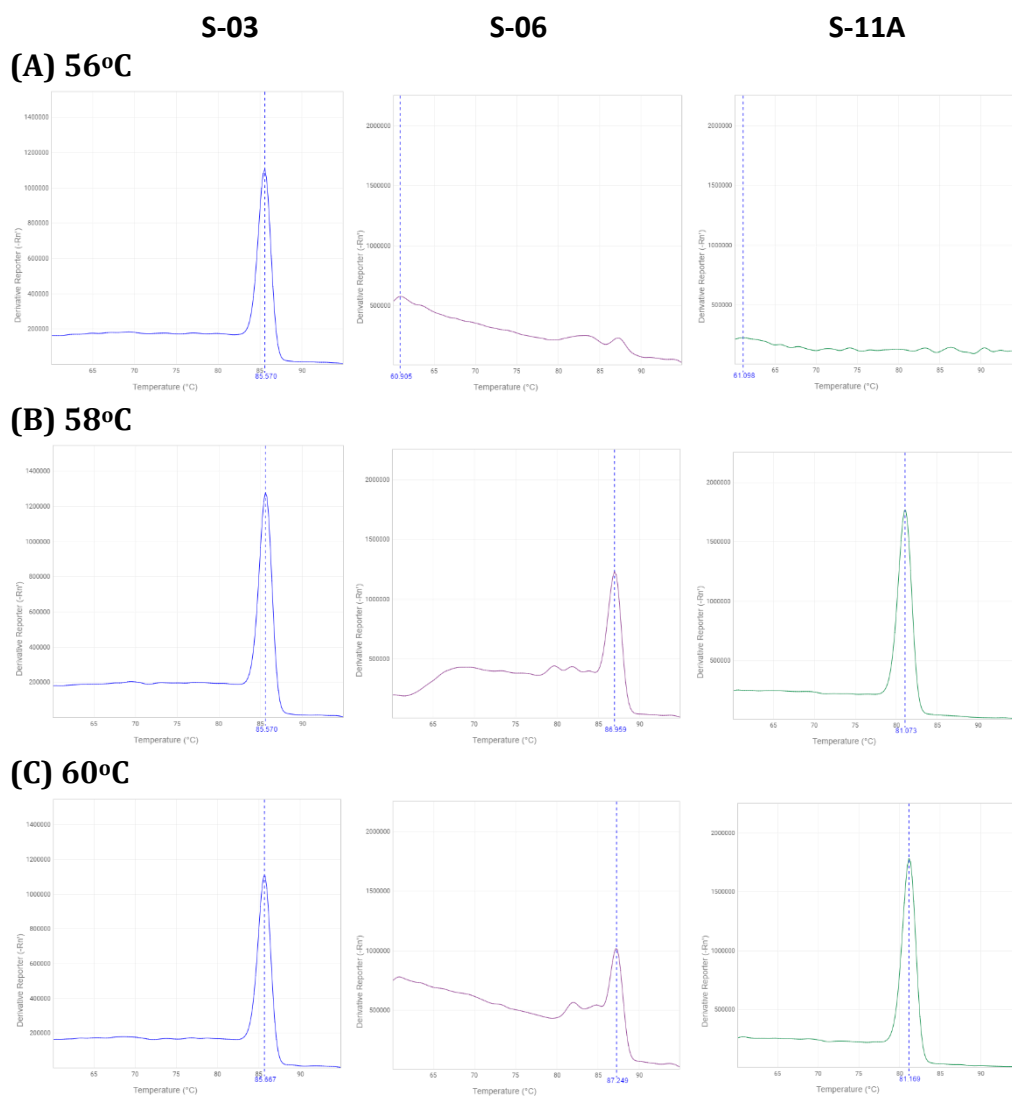
Figure C.I continued.

(D) 62ºC
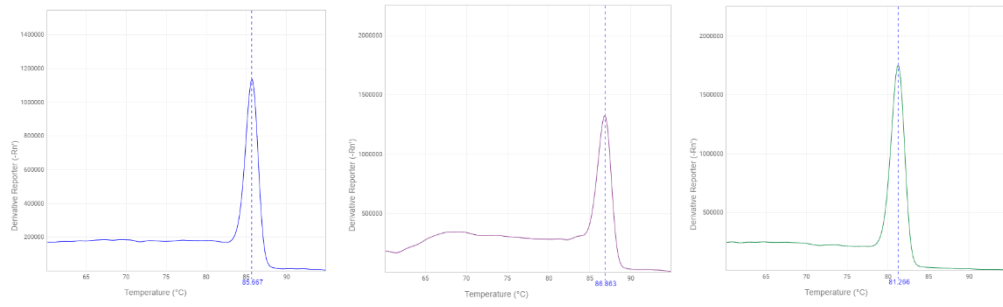
(E) 64°C
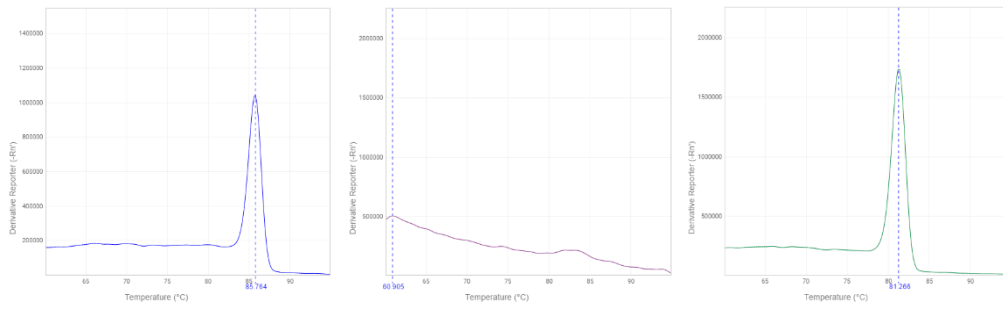
(F) 66°C
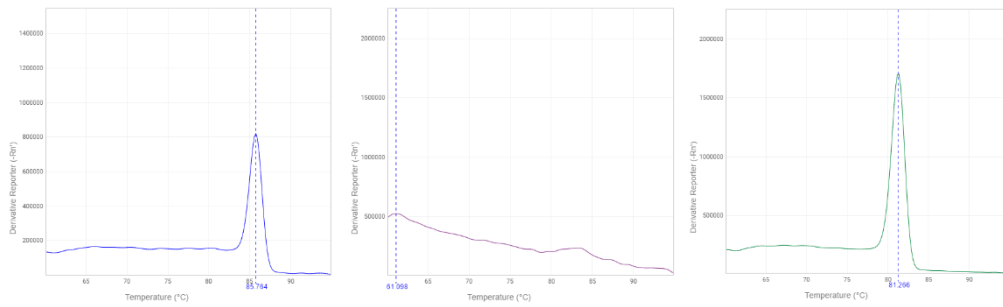
Figure C.II S-03 q-PCR primer annealing temperature gradient dissociation curves for serial dilution and negative samples. Primer annealing temperatures: (A) 62°C, (B) 63°C and (C) 64°C. Serial dilutions A: 100%, B: 10%, C: 1%, D: 0.1%, E: 0.01% and F: 0.001%) were pipetted from a starting quantity of 200ng. Negative controls included were G: DNA negative for the target of interest and H: non-template water control. Amplicons were continuously heated from 55^0C - 95^0C and temperature (^{0}C) was plotted against the derivative Reporter (-Rn') to determine the amplicon melting temperature (T_m). The dissociation curves indicated the presence of a specific amplicon for serial dilutions over 5 logs at a primer annealing of 63°C only and this temperature was selected for all future optimisations and experiments.

(A) 62°C   **(B) 63°C**   **(C) 64°C**
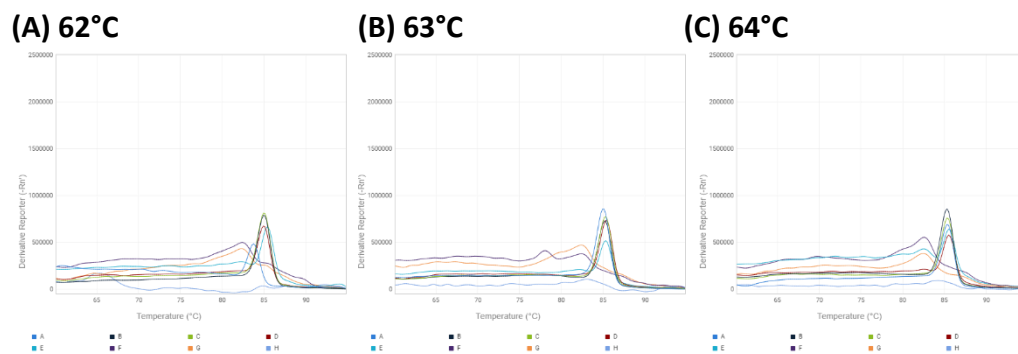
Figure C.III HCK 63°C primer annealing q-PCR dissociation curve
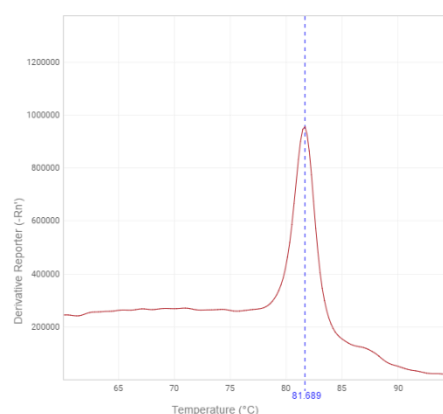
Table C.II InDel primer concentration optimisation by comparison of q-PCR amplification efficiencies. Standard curves were produced from artificial spiking experiments whereby DNA positive for the polymorphic target of interest was diluted in DNA negative for the target. Serial 1:10 dilutions over five logs (100%, 10%, 1%, 0.1% and 0.01%) were pipetted from a starting quantity of 200ng and an additional 0.05% dilution was also included. q-PCR reactions for each dilution and target were set up in duplicate containing a variable amount of forward and reverse HLA primer. **Red** indicates amplification efficiency closest to 100% and thus the optimal primer concentration selected for future experiments.

Target	PCR efficiency (%) at each primer concentration tested		
	4.5 pmol	**3.0 pmol**	**1.5 pmol**
S-03	**99**	99	83
S-06	103	**98**	87
S-11A	85	**99**	90

Table C.III HCK primer concentration optimisation by comparison of q-PCR amplification efficiencies. Standard curves were produced from artificial spiking experiments whereby DNA was diluted in water. Serial 1:2 dilutions over six logs were pipetted from a starting quantity of 200ng. q-PCR reactions for each dilution and target were set up in duplicate containing a variable amount of forward and reverse HLA primer. **Red** indicates amplification efficiency closest to 100% and thus the optimal primer concentration selected for future experiments.

| Primer concentration (pmol)	PCR efficiency (%)
1.50	122
1.00	**109**
0.75	133
0.50	-

Table C.IV Sensitivity of each InDel q-PCR assay. Standard curves were produced from artificial spiking experiments whereby DNA positive for the polymorphic target of interest was diluted in DNA negative for the target. Serial dilutions (10%, 1%, 0.1%, 0.05%, 0.01% and 0.005%) were pipetted from a starting quantity of 200ng. q-PCR reactions for each dilution and target were set up with five replicates. The linear dynamic range (LDR) was the last point where the curve maintained linearity, defined as a coefficient of determination (R^2) $\geq$ 0.99.

| Target	LDR (%)	Positive replicates
S-03	0.01	5/5
S-06	0.01	5/5
S-11A	0.01	5/5

Figure C.IV Representative InDel q-PCR amplification plots (1) and standard curves (2). (A) S-03, **(B)** S-06 and **(C)** S-11A. Standard curves were produced from artificial spiking experiments whereby DNA positive for the HLA target of interest was diluted in DNA negative for the target. Serial dilutions (100%, 10%, 1%, 0.1%, 0.05% and 0.01%) were pipetted from a starting quantity of 200ng and set up in triplicate.
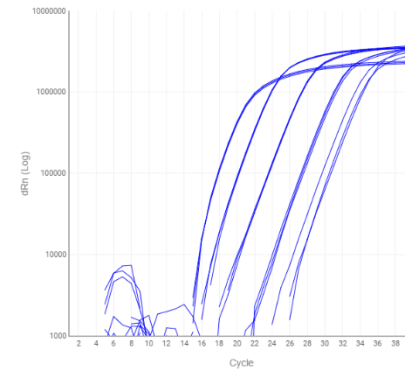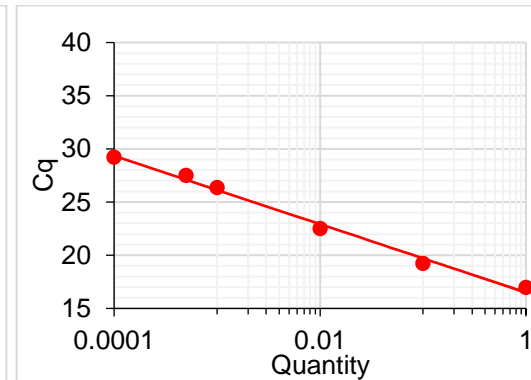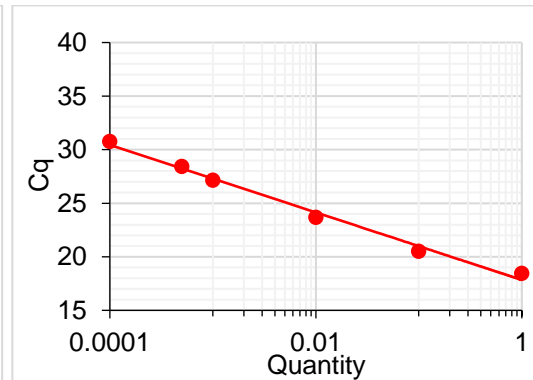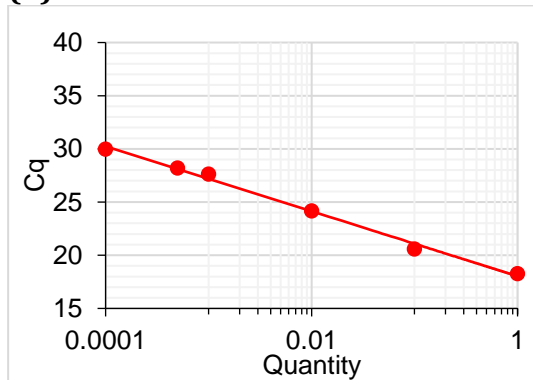
(A) S-03
(1)
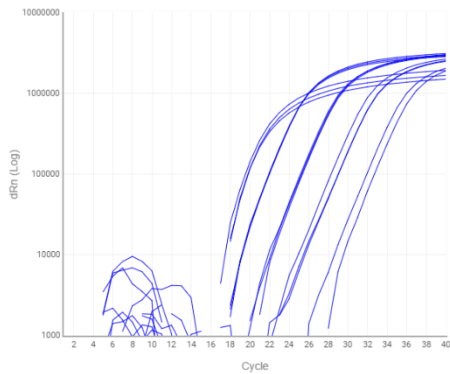
(B) S-06
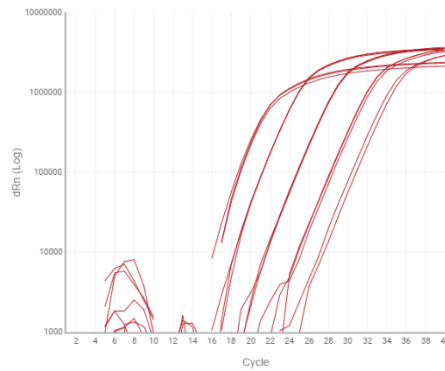
(C) S-11A

(2)

Table C.V InDel q-PCR quantitation cycle (C$_q$) results for representative standard curves. Standard curves were produced from artificial spiking experiments whereby DNA positive for the polymorphic target of interest was diluted in DNA negative for the target. Serial dilutions (100%, 10%, 1%, 0.1%, 0.05% and 0.01%) were pipetted from a starting quantity of 200ng and set up in triplicate. C$_q$ values are shown as the mean ± standard deviation (S.D.) for three replicates.

Target	Mean C$_q$ ± S.D. for each target quantity					
	100%	**10%**	**1%**	**0.1%**	**0.05%**	**0.01%**
S-03	18.262 ± 0.156	20.578 ± 0.023	24.168 ± 0.045	27.617 ± 0.148	28.187 ± 0.119	29.953 ± 0.377
S-06	18.444 ± 0.175	20.505 ± 0.017	23.673 ± 0.099	27.144 ± 0.409	28.415 ± 0.104	30.757 ± 0.585
S-11A	16.956 ± 0.037	19.217 ± 0.066	22.497 ± 0.049	26.345 ± 0.131	27.502 ± 0.290	29.198 ± 0.555

Table C.VI Representative InDel q-PCR standard curves properties.
Standard curves were produced from artificial spiking experiments whereby
DNA positive for the polymorphic target of interest was diluted in DNA negative
for the target. Serial dilutions (100%, 10%, 1%, 0.1%, 0.05% and 0.01%) were
pipetted from a starting quantity of 200ng and set up in triplicate.

Target	Slope	Intercept	R^2
S-03	-3.047	18.083	0.993
S-06	-3.110	17.937	0.990
S-11A	-3.213	16.497	0.990

**Table C.VII Informativity of InDel polymorphisms for NIMA in the CBU and
maternal pairs evaluated.**

	Total genotyped	Informativity	
		Informative	Non-informative
S-03	66	8 (12)	58 (88)
S-06	66	14 (21)	52 (79)
S-11A	66	13 (20)	53 (80)

Appendix D – Peer reviewed publications

L. Powley, C. Brown, A. Melis, Y. Li, G. Parkes and C. Navarrete (2016) Consideration of Noninherited maternal antigens as permissible HLA MMs in cord blood donor selection. *Bone Marrow Transplantation* **51**: 675 – 679. doi: 10.1038/bmt.2015.344

Appendix D – Peer reviewed publications

Appendix E – Conferences and other meetings

L. Powley Noninherited maternal antigens in HSCT. *Invited speaker at BSHI HSCT Special Interest Group, 2017, Birmingham.*

L. Powley, Y. Li, G. Parkes, C. Brown and C. Navarrete. Consideration of Noninherited Maternal Antigens as Permissible MMs in Cord Blood Unit Donor Selection. *Poster presentation at the BSHI Annual Conference, 2015, Cambridge.*

L. Powley, Y. Li, C. Brown and C. Navarrete. Consideration of Noninherited Maternal Antigens in Cord Blood Unit donor selection to improve the availability of HLA matched cord blood donors for patients from a diverse ethnic background. *Oral presentation at the Division of Infection and Immunity PhD Colloquium 2015, UCL.*

L. Powley, Y. Li, G. Parkes, C. Brown and C. Navarrete. Consideration of Noninherited Maternal Antigens as Permissible MMs in Cord Blood Unit Donor Selection Increases the Donor Pool Available to Patients Requiring Cord Blood Transplantation. *Poster presentation at the World Cord Blood Congress V and Innovative Cell Therapies, 2015, Monaco.*

L. Powley. NIMA in HSCT. *Oral presentation at 11th International Summer School for Immunogenetics, Colorado, 2014.*

L. Powley, C. Brown, A. Anand, Y. Li, G. Parkes and C. Navarrete. Expansion of the HLA virtual phenotypes of the NHS-CBB units by using noninherited maternal antigens. *Poster presentation at 10th International Donor Registry Conference and WMDA Working Group Meetings, 2014, London*

L. Powley, C. Brown, A. Anand, Y. Li, G. Parkes and C. Navarrete. Expansion of the HLA virtual phenotypes of the NHS-CBB units by using noninherited maternal antigens. *Poster presentation at NHSBT R&D Conference, 2013.*

Appendix E – Conferences and other meetings

Appendix F – Travel grants

BSHI Travel Bursary – To attend World Cord Blood Congress V and Innovative Cell Therapies, Monaco 2015 (£400)

Netcord Travel Award – To attend World Cord Blood Congress V and Innovative Cell Therapies, Monaco 2015 (€1,000)

BSI Travel Bursary – To attend 11th International Summer School for Immunogenetics, Colorado, USA 2014 (£800)

EFI Bursary – To attend 11th International Summer School for Immunogenetics, Colorado, USA 2014 (€750)