Killer-cell immunoglobulin-like receptor genomics and haematopoietic progenitor cell transplantation: Utilising allelic polymorphism within donor selection algorithms

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Abstract

The highly polymorphic killer-cell immunoglobulin receptor (KIR) genes encode some of the key receptors involved in regulation of the cytotoxic action of natural killer (NK) cells, facilitating clearance of virally infected and neoplastic cells.

To investigate how the KIR complex effects the outcome of selected haematopoietic progenitor cell transplantation (HPCT) programmes, next generation sequencing was used to evaluate allelic polymorphism of KIR, in a single transplant centre cohort (n=281), to construct a refined model for transplant donor selection. The viability of more cost- effective donor KIR assessments was also explored. High levels of KIR diversity in both gene content and allelic polymorphism was evident, and no KIR donor selection model studied influenced 3-year overall survival event-free survival or reduce relapse incidence in the cohort (n=115).

The impact of allelic polymorphism of KIR2DL1, 2DL2, and 2DL3 upon post-transplant outcomes was investigated in two patient groups: Cohort A (KIR2DL1 n=113, KIR2DL2/3 n=114) encompassing all transplants in the sample, and Cohort B (KIR2DL1 n=86, KIR2DL2/3 n=87), a sub-cohort of T cell depleted reduced intensity conditioning transplants. Close linkage between the allele groups of KIR2DL1, 2DL2, and 2DL3 negated the value of independent analysis into KIR2DL1 and 2DL3, and the KIR2DL2*001 group was identified as acting as a weak proxy for KIR B/x diplotype profiles (present in 76.6% of KIR B/x donors). Examination of three distinct donor KIR2DL1 allele groups (KIR2DL1*001/002, 003, and 004) identified KIR2DL1*003 positive donors as detrimental to post-transplant outcomes, reducing 3-year overall survival (OS) (36.5% vs 61.8%, p=0.046, p_c=0.184) and event-free survival (EFS) (28.9% vs 58.8%, p=0.015, p_c=0.084), and increasing 3-year relapse incidence (54.1% vs 21.9%, p=0.019, p_c=0.090). This data suggests KIR2DL1*003 positive donors should be avoided in the setting of T cell depleted reduced intensity conditioning HPCT.

The described cohort data was further interrogated to investigate the clinical value of KIR2DL1 signature single motif (codon 114 and 245) assessments, with the aim of reducing the financial cost of testing for these donor characteristics. This showed that donor KIR2DL1 with proline at position 114 (KIR2DL1-P^{114}) provided improved OS (58.8% versus 28.6%, p=0.008, p_c=0.032), EFS (51.0% versus 25.7%, p=0.018, p_c=0.060), and reduced incidence of relapse (31.5% versus 54.9%, p=0.028, p_c=0.084) compared to transplants using KIR2DL1-P^{114} negative donors. In contrast, transplants where donors possessed leucine at position 114 (KIR2DL1-L^{114}) exhibited decreased 3-year OS (36.5% versus 61.8%, p=0.046, p_c=0.138) and EFS (28.9% versus 58.8%, p=0.015, p_c=0.060), combined with increased relapse rate (54.1% versus 21.9%, p=0.019, p_c=0.076). Position 245 (a cysteine/arginine dimorphism) appeared less influential in transplant outcomes, with a weak trend seen for beneficial outcomes in KIR2DL-C^{245} likely attributed to its association with KIR2DL1-P^{114}. KIR2DL1-R^{245} in the donor was found to be neutral with no influence upon post-transplant outcomes.

These findings provide proof of principle that position 114 of KIR2DL1 may be a viable donor selection mechanism, and confirms the proposition that KIR genomics should be assessed in the context of wider transplant conditioning protocols. A larger multi-centre study is recommended to verify results, and to assess the applicability in other transplant centres.

Lay Abstract

Haematopoietic (derived from the Greek words for “Blood” and “to make”) stem cells have the ability to develop into all cells of the blood system, and so a transplant of these cells can provide the recipient with a healthy source of blood cells. For individuals suffering from leukaemia, a severe cancer of the blood system, this represents the only form of a cure for these patients.

As the transplant delivers the complete blood system of the donor, the recipient also inherits the donor’s immune system. It is essential that the donor’s immune system accepts the recipient’s body as self to minimise the risk of the transplant failing. For this reason, the donor and recipient immune system must be compatible to minimise risk of transplant failure and maximise the likelihood of clearing cancer cells.

The immune system is highly complex, and so it is not completely understood how the donor immune system can be harnessed to increase the success of the transplant. Natural killer (NK) cells are a type of immune cell that may help improve transplant outcomes due to their ability to kill cancer cells and cells infected by viruses. To do this, NK cells use Killer-cell Immunoglobulin-like Receptors (KIR) on their surface to detect unhealthy cells to be killed. Recent developments in gene sequencing have allowed us to investigate the blueprints of the KIR receptors – the KIR genes – in greater detail than ever before. Researching these genes may help to define beneficial aspects of transplant donors to help clinical teams select the best possible donor with the highest likelihood of a successful transplant.

This study explores the variation of KIR genes in a local Manchester population, and examines the effectiveness of current models used for KIR gene assessment in transplant donors. Analysis then focussed upon one particular KIR gene, KIR2DL1, looking at three different types of this receptor: KIR2DL1*001/002, 003, and 004 groups. Donors positive for KIR2DL1*003 group gene were found to suffer poor transplant outcomes, with an increased risk of cancer relapse and poor survival. Donors with this genetic characteristic should therefore be avoided. Closer analysis of KIR2DL1 found a single part of the gene, involved in the KIR receptor binding the target cell, had a large influence upon the transplant outcome.

Combined, this data contributes to our understanding of what makes an ideal transplant donor to improve patient care in leukaemia patients.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

Preface

I have worked in clinical Histocompatibility & Immunogenetics (H&I) for the past ten years, in service of both the local haematopoietic progenitor cell transplantation and solid organ transplantation programmes at the Manchester University NHS Foundation Trust. I treasure this vocation greatly, having pursued every opportunity to be deeper involved in this great community of scientists and healthcare staff.

The seed of this thesis germinated circa 2015, shortly after commencing the NHS Higher Specialist Scientific Training (HSST) programme as part of the first cohort for this novel curriculum. At this time, next generation sequence (NGS) was a relatively new tool to clinical H&I, with many transplant centres around the United Kingdom implementing NGS for HLA genotyping in transplantation programmes. This exciting new technology made sequencing genes of interest viable at medium throughput, allowing us to collect high-resolution genotyping results at a higher rate than was previously viable. This led us to speculate about other genes that could potentially be sequenced with this technique to investigate transplantation from a novel perspective. The KIR genes were the obvious choice, with several clinical models available for KIR gene presence/absence, but little information on the influence of KIR allelic polymorphism. This turned out to be a fruitful area of research, with the KIR field of research rapidly expanding in understanding and knowledge as we developed our study protocol and hypothesis.

With the time pressures and financial limitations involved in a part-time doctorate work, I unfortunately did not have the resources to develop and validate an in-house NGS kit. Our initial ventures with commercial custom kits designed by automated algorithms were found to be disastrously unsuccessful, owing to the complexities of KIR genetics, and the high homology between the different KIR genes. Fortuitously, we were able to collaborate with GenDx, an organisation who were in the progress of developing the first commercially available KIR NGS kit, and were seeking to test out the kit on a clinical cohort. Without this collaboration, the following thesis would not have been possible.

The findings of my thesis were far more exciting than the trivial academic exercise I had initially anticipated. Furthermore, plenty more sequencing data was generated than I could possibly fit into the format of this thesis. This thesis signifies the beginning of a journey I hope will continue long into the future.

Dedicated to

Phyllis & Jack

Beryl (& Thomas)

Acknowledgements

My thanks and appreciation to my supervisors, Stephen Sheldon (Clinical Supervisor) and Lynne Hampson (Academic Supervisor), for their diligence, guidance and support throughout the project. My wider thanks to Kay Poulton, Natalia Diaz Burlinson, Helena Lee, and all at the Transplantation Laboratory of the Manchester University NHS Foundation Trust, for the frequent and overwhelmingly generous flexibility offered to allow me to study and carry out this research.

Thank you for the kind assistance of:

Loes van de Pasch, Sanne Vendelbosch, and GenDx for providing the NGS kits for sequencing of KIR genes and the accompanying NGS Go analysis software, as well as guidance in use of this kit and software.

Katie Kichula, Nicholas Pollocks, and Paul Norman at the Anschutz Medical Campus, University of Colorado, for carrying out capture NGS to verify identification of novel KIR alleles.

Catherine Fullwood for advising upon the statistics used in this thesis.

Steven Marsh and William Bultitude of Anthony Nolan, for providing DNA samples with defined KIR alleles. These samples were invaluable for validating our testing.

Rebecca McGuire for her assiduous aid in retrieving the study samples from frozen storage.

Abbreviations

95% CI = 95% Confidence Interval

aKIR = Activating killer-cell immunoglobulin receptor

ABSR = Amplicon-based short read

CenA / CenB = Centromeric region of KIR A/B haplotype

CMV = Cytomegalovirus

CNV = Copy number variation

EFS = Event-free survival

GvHD = Graft versus host disease

HBTE = Hybridisation-based target enrichment

HLA = Human leukocyte antigen

HPC = Haematopoietic progenitor cell

HPCT = Haematopoietic progenitor cell transplantation

HR = Hazard ratio

iKIR = Inhibitory killer-cell immunoglobulin receptor

iNKT cell = Invariant natural killer T cell

KIR = Killer-cell immunoglobulin receptor

KIR2DL1-P^{114} / -L^{114} = KIR2DL1 receptors possessing proline/ leucine at position 114

KIR2DL1-C^{245} / -R^{245} = KIR2DL1 receptors possessing cysteine/arginine at position 245

MAC = Myeloablative conditioning

NaOH = Sodium hydroxide

NGS = Next generation sequencing

NK cell = Natural killer cell

NRM = Non-relapse mortality

OS = Overall survival

PCR = Polymerase chain reaction

PCR-SSP = Polymerase chain reaction sequence-specific primers

PCR-SSO = Polymerase chain reaction sequence-specific oligonucleotides

qPCR = Real-time polymerase chain reaction

Q30 = Quality score of 30

RIC = Reduced intensity conditioning

RSB = Resuspension buffer

SPB = Sample purification beads

TcR = T cell receptor

TBE buffer = Tris-borate-EDTA buffer

TE buffer = Tris-EDTA buffer

TelA / TelB = Telomeric region of KIR A/B haplotype

Units of Measurement

µL = Microlitres

°C = Degrees celsius

kb = kilobases

M = Molar

mM = Micromolar

mA = Milliamps

min = Minutes

ng = Nanograms

ng/µL = Nanograms per microlitre

RCF = Relative centrifugal force

rpm = Rotations per minute

s = Seconds

V = Volts

Chapter 1 | Current perspectives of the genomics and proteomics of killer-cell immunoglobulin-like receptor in haematopoietic progenitor cell transplantation (Literature Review)

Summary

Natural killer cells preferentially target and kill malignant and virally infected cells. Both these properties present compelling clinical utility in the field of haematopoietic progenitor cell transplantation (HPCT), potentially promoting a graft versus leukaemia effect in the absence of graft versus host disease and protecting against cytomegalovirus activation. Killer Ig-like receptors (KIR) play a central role in the cytotoxic action of natural killer cells, providing opportunity for improving transplantation outcomes by prioritising potential donors with optimal characteristics. Numerous algorithms for assessing KIR gene content as part of HPCT donor selection protocols exist, but no single model has been found to be universally applicable in all transplant centres. This review summarises several of the predominant strategies in KIR assessment algorithms, discussing their basic scientific principles, clinical utility, and benefits to post-transplant outcomes. Finally, the review will consider how future donor selection protocols could develop towards unifying the concepts of KIR proteomics and genetics for optimising patient care.

1.1 Introduction

The cytotoxic activity of the Natural Killer (NK) cell delivers an innate immune defence against infection and malignancy. In recognising the absence of HLA class I expressed on the surface of the target cell, NK cells are capable of recognising abnormal cells for clearance that would otherwise be missed by adaptive immune cells (Kärre et al., 1986). To this end, the specificities of the NK cell's CD94/NKG2A and Killer Immunoglobulin-like Receptors (KIR) for HLA class I are central to this immunosurveillance activity. CD94/NKG2A is a C-type lectin receptor capable of binding HLA-E, a constitutively expressed non-classical HLA class I protein complex that is presented on the surface of nucleated cells as part of endogenous antigen presentation. The KIR glycoproteins provide a highly specific form of recognition

that is proficient in detecting down-regulation of a single HLA class I allotype, with affinity for the C1, C2, Bw4 or A3/11 epitopes present within some HLA class I protein complexes (Colonna et al., 1992; Colonna et al., 1993; Cella et al., 1994; Mandelboim et al., 1996; Hansasuta et al., 2004; Moesta et al., 2008; Hilton et al., 2015a; Saunders et al., 2015).

The genetic complexities of KIR gene cluster have become an area of increasing interest to the Histocompatibility and Immunogenetics (H&I) community, with KIR assessment algorithms incorporated into haematopoietic progenitor cell transplantation (HPCT) programmes to provide clinically significant improvements in post-transplant outcomes. The advent of next generation sequencing (NGS) now offers the opportunity for research and clinical laboratories to investigate the polymorphism of KIR genes in far greater detail, with the ultimate goal of developing a clinical utility for this molecular data in donor selection algorithms. This review will consider the current understanding of KIR proteomics and genetics in the context of the current and future utility of KIR analysis in HPCT donor selection algorithms.

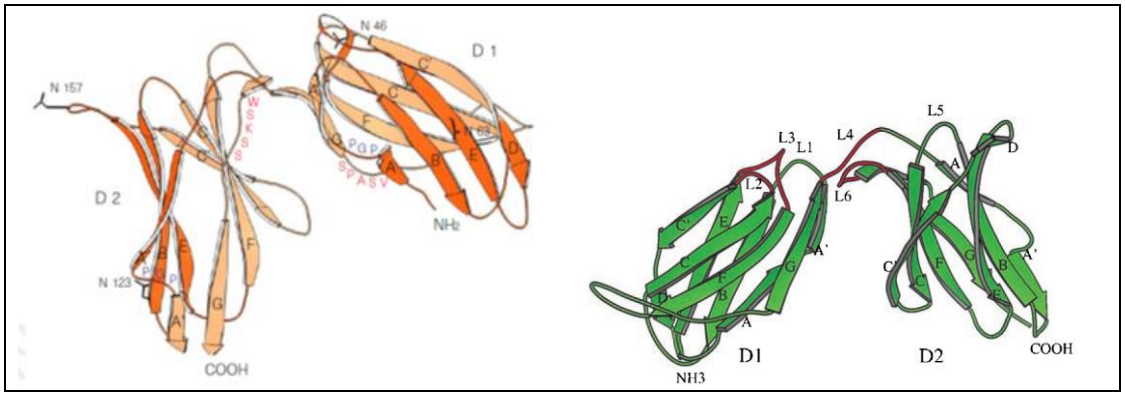


Figure 1 – Tertiary structure of KIR2DL1 and 2DL2

Ribbon diagram of KIR2DL1 (left) and KIR2DL2 (right). The KIR2DL1 binding regions (orange) of the D1 and D2 domains are clearly visible (images from Fan et al 1997 and Snyder et al, 1999).

1.2 KIR Proteomics

1.2.1 Proteomic Structure

One of the first KIR glycoproteins to be described at the molecular level was KIR2DL1 (Fan et al., 1996; Fan et al., 1997). The ABED (residues 10-13, 24-30, 60-66, and 54-55) and ABE (residues 109-113, 123-130, and 160-168) anti-parallel β-pleated sheets of KIR2DL1's D1 and D2 domains respectively form the ligand binding site, with the C'CFGAA' anti-parallel β-pleated sheets of both domains forming the remaining extracellular structure (Figure 1) (Fan et al., 1997). The two extracellular domains are connected by the L4 loop (residues 101-108), with residues 102-104 forming a helical conformation that brings the two domains tightly together (Fan et al., 1997). The D1 and D2 domains are also associated via VSAPS (residues 90-94) and WSKSS (residues 188-192) motifs within loops of the respective domains (Fan et al., 1997).

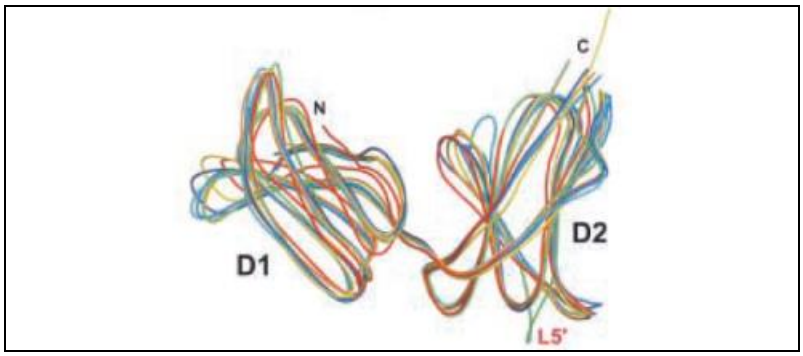


Figure 2 – Structural overlay of the D1 and D2 domains of various KIR2D glycoproteins, demonstrating the high structural homology between these receptors

The KIR glycoproteins presented are as follows: KIR2DL1, red; KIR2DL2 (trigonal form), dark blue; KIR2DL2 (orthorhombic form), cyan; KIR2DL2 (free), marine blue; KIR2DL2 (bound to HLA-Cw3), orange; KIR2DL3, yellow; KIR2DS2, green (image from Saulquin et al, 2003).

KIR2DL2 and 2DL3 broadly share structural homology with KIR2DL1 in their extracellular domains (Figure 1, Figure 2) (Snyder et al., 1999; Saulquin et al., 2003).

The hinge joint angle between D1 and D2 domains of the free KIR receptor is variable between genes: approximately 55°, 84°, and 80° for KIR2DL1, 2DL2 and 2DL3, respectively (Fan et al., 2001; Frazier et al., 2013). Upon binding to its cognate ligand, the hinge opens, with the most extensive alteration observed in KIR2DL1 opening to 66° (Fan et al., 2001). KIR2DS2 also shares close extracellular domain structural homology with the inhibitory KIR2D receptors, with the D1 and D2 domains solely differing from KIR2DL3 at two residues (Figure 2, Figure 3).

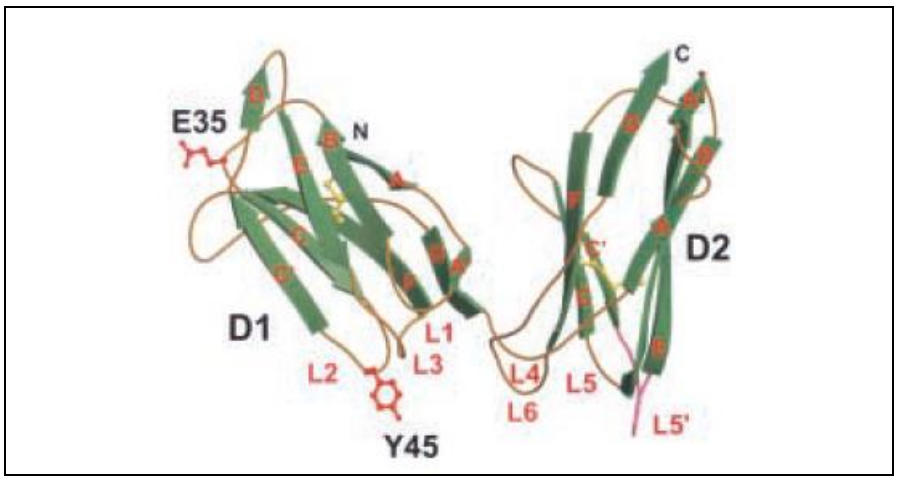


Figure 3 — Tertiary structure of KIR2DS2

In secondary structure of the extracellular domains, KIR2DS2 differs from KIR2DL3 at only two residues within the D1 domain: tyrosine at pos45 (Y45) and glutamic acid at pos35 (E35). These two positions are highlighted in red (image from Saulquin et al, 2003).

KIR3DL1 possesses three extracellular domains: D0, D1, and D2, with all three domains contributing to ligand interactions (Figure 5) (Vivian et al., 2011). The hinge between the D0 and D1 domains is similar to that between D1 and D2 (83° and 81° respectively) (Vivian et al., 2011).

1.2.2 Inhibitory KIR Interactions

The "Missing Self" model helped expound the mechanism by which NK cells selectively eliminate target cells, demonstrating a correlation between the reduction of MHC class I expression and an inability to inhibit the cytolytic action of NK cells in

a murine model (Kärre et al., 1986; Ciccone et al., 1995). In humans, NK cells bind to an array of HLA class I epitopes, with a majority of these interactions involving position 80 within the α_1 domain of HLA-Cw (Colonna et al., 1992; Colonna et al., 1993; Mandelboim et al., 1996; Pende et al., 2019). Approximately half of all HLA-Cw antigens contain an asparagine residue at position 80, while the remainder contain a lysine residue, categorised as C1 and C2 epitopes respectively (Winter et al., 1998; Robinson et al., 2015). Most inhibitory KIR receptors are highly specific for one of these epitopes. For example, most KIR2DL2 and 2DL3 receptors bind the C1 epitope (though some display weak affinity for the C2 epitope and HLA-B antigens), and most KIR2DL1 receptors bind the C2 epitope (Moesta et al., 2008; Hilton et al., 2015a). Other KIR/HLA interactions include KIR3DL1 exhibiting specificity for the HLA Bw4 epitope, and KIR3DL2 binding the HLA-A3/A11 epitope found on HLA-A3 and A11 complexes (Hansasuta et al., 2004; Saunders et al., 2015). Despite the close functional relationship between KIR proteins and their HLA class I ligands, the two gene systems are located on separate chromosomes, 19q13.4 and 6p21.1 respectively. Though meiotic division should largely negate the strength of co-evolution between two gene systems located on disparate chromosomes, there is evidence that KIR and HLA have co-evolved to maximise interactions (Parham et al., 2012; Gendzekhadze et al., 2015; Hilton et al., 2015a; Wroblewski et al., 2019).

The initial investigations into the proteomics of KIR2DL1 considered its interactions with HLA-Cw4 (Fan et al., 1996; Fan et al., 1997). Interactions with the positions 44, 71, 72, and 183 of KIR2DL1 were first defined, with position 44 presenting the primary residue for interaction with the C2 epitope (Fan et al., 1997). Several of the loops of KIR2DL1 were also found to interact with HLA class I, with L2 (residues 43-47) and L3 (residues 66-75) loops of the D1 domain and L6 (residues 180-187)

loop of the D2 domain involved in ligand binding (Fan et al., 1997). The allo-specificity of KIR2DL2 and 2DL3 for the C1 epitope is also determined by position 44, with lysine found at this residue (Boyington et al., 2000). Consequently, exchanging methionine for lysine at position 44 of KIR2DL1 converts the receptor's specificity from the C2 epitope to the C1 epitope (Winter and Long, 1997).

The C1/C2 epitope (residue 80) of HLA-Cw is found at the COOH proximal terminus of the α1 helix of the HLA-C molecule (Fan and Wiley, 1999). It is of note that a majority of the HLA-Cw hyper-variation observed between alleles occurs within the peptide binding groove (Fan and Wiley, 1999; Robinson et al., 2020). Once a peptide is bound within the peptide binding groove, position 80 is one of the few points of variation that remains exposed on the surface of the HLA-Cw molecule (Fan and Wiley, 1999). The region of the HLA-Cw complex surrounding the exposed position 80 residue also holds a complementary positive polarity to KIR2DL1's negative polarity, promoting KIR interactions (Fan et al., 1996; Fan and Wiley, 1999).

Binding of KIR to HLA class I is dependent upon the peptide bound to the HLA complex. The HLA-Cw peptide's P7 and P8 side chains have been demonstrated to influence KIR interaction, with negatively charged P7 side chain impeding KIR2DL1 binding (Rajagopalan and Long, 1997). KIR2DL2/3 is more restrictive in regards to peptide recognition, as the P7 and P8 side chain of the peptide reach towards the KIR in KIR2DL2/HLA-Cw10 interactions (Figure 4) (Boyington et al., 2000). P2 and P10 of the presented peptide also contribute towards KIR2DL2/3 avidity (Sim et al., 2017). In contrast, the P7 and P8 side chains are not exposed in KIR2DL1/HLA-Cw4 interactions, and so the peptide sequence is less influential in binding avidity (Fan et al., 2001). Ultimately, the contribution of the presented peptide to KIR interactions

results in KIR2DL2/3 binding avidity generally being weaker than that of KIR2DL1 (Sim et al., 2017).

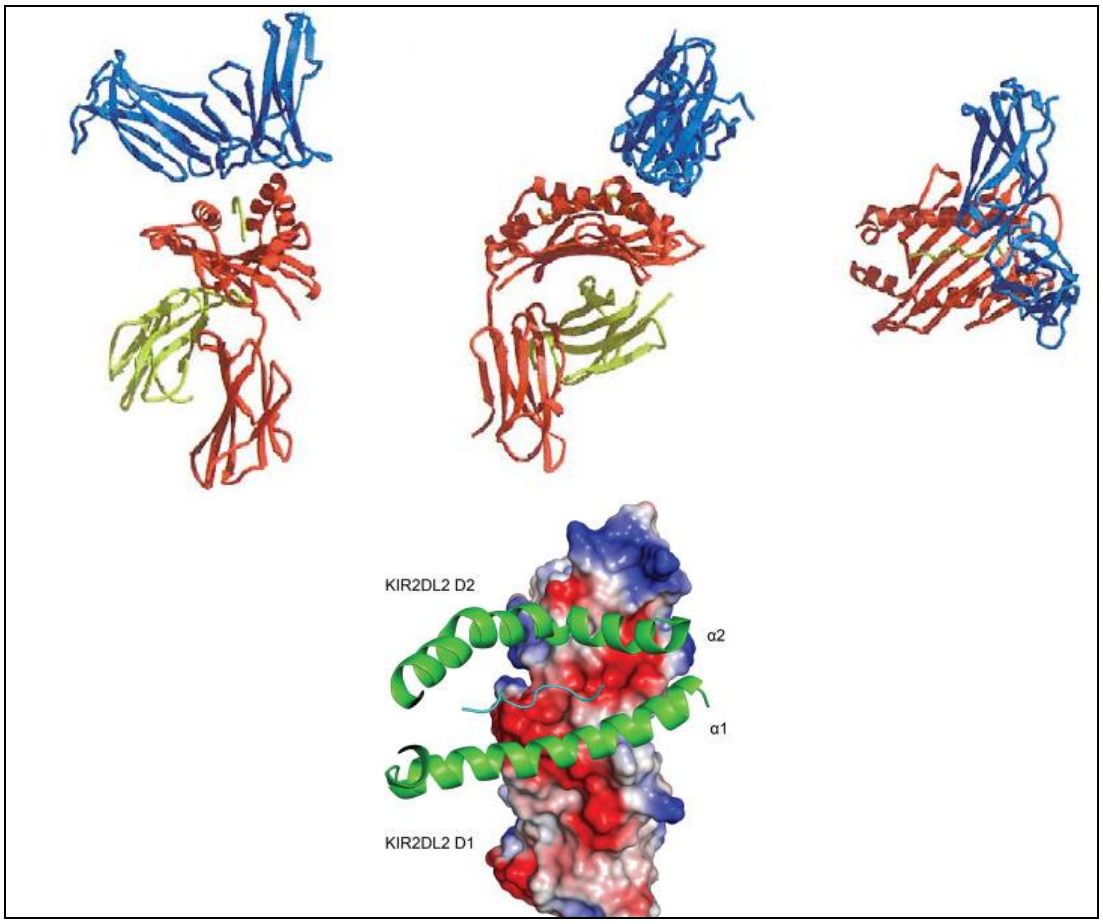


Figure 4 – Structural diagrams demonstrating the interactions of KIR2DL2 and HLA-Cw3 (with peptide loaded) (top row) KIR2DL2 (blue) bind HLA-Cw3 (heavy chain red, β_2-microglobulin yellow) across the proximal end of the HLA, across the peptide binding groove and the peptide itself (yellow). View from front (left), side (centre), and top (right) (image from Boyington & Sun, 2002). (bottom row) A closer look at the binding of KIR2DL2 and the binding domain of HLA-Cw3, including electrostatic potential of KIR2DL2 surface (red = negative, blue = positive, white = neutral) (image from Chen et al, 2009).

All three extracellular domains of KIR3DL1 contribute to interactions with the HLA-Bw4 ligand (Figure 5). The D0 domain lies perpendicular to the antigen binding cleft, making contact with the side of the HLA. The D1 and D2 domains lie across the peptide binding cleft and bound peptide, forming a majority of the buried surface area (Vivian et al., 2011). The D1 domain interacts with the Bw4 epitope at position 80 of the relevant HLA class I, with position 79, 80, and 83 of the HLA interacting

with position 138/140 (glycine/serine), 166 (leucine), and 278 (histidine) of KIR3DL1, respectively (Vivian et al., 2011). Despite the Bw4 epitope representing the focal point of KIR3DL1 allo-specificity, interactions between the three KIR3DL1 domains and HLA class I occur across the buried surface (Cella et al., 1994; Vivian et al., 2011).

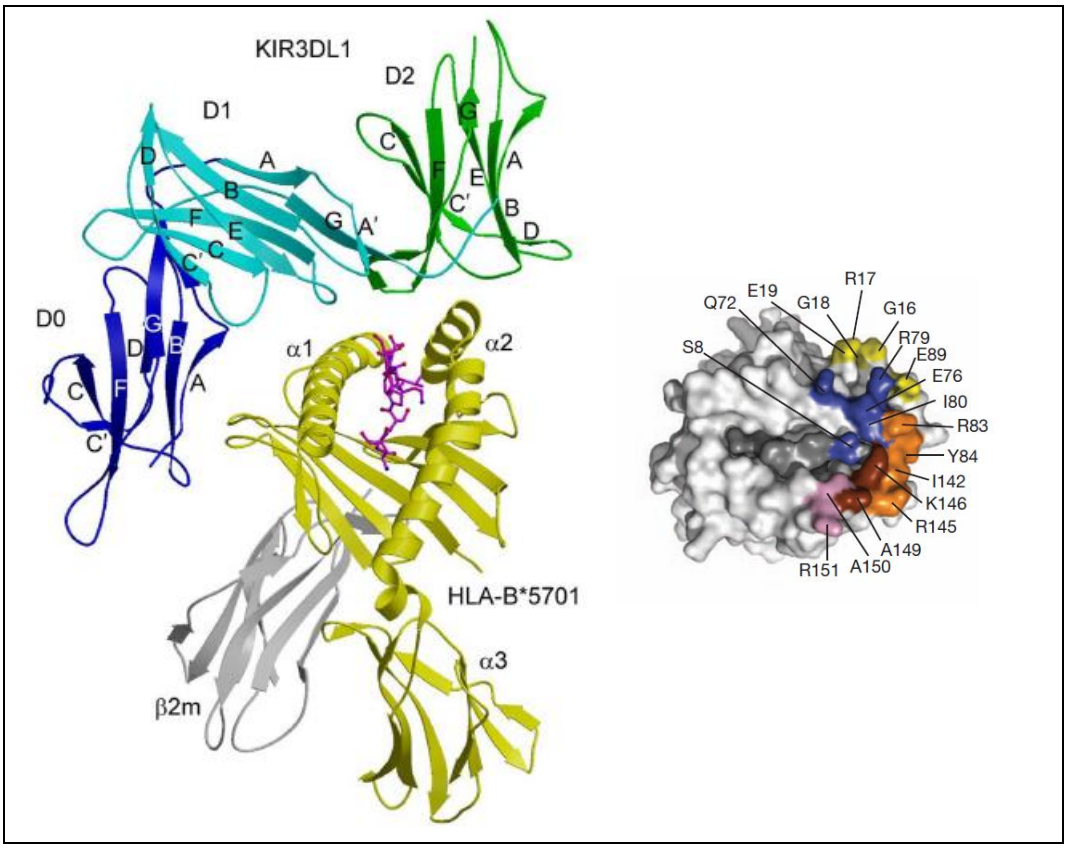


Figure 5 – Structural diagram of interactions between KIR3DL1 and HLA-B57 (with peptide loaded) (left) and spatial diagram of the HLA-B57 surface demonstrating the points of interaction with KIR3DL1 (right).
Left: KIR3DL1 binds to HLA class I via C0 (dark blue), D1 (cyan), and D2 (green) domains, across the proximal end of the HLA complex (yellow, with white β_2-microglobulin) and the peptide (magenta) (image from Li et al, 2014). Right: Surface of HLA-B57, displaying points of interaction with the KIR3DL1 D0 (yellow), D1 (blue), D1/D2 loop (pink), and D2 (orange) domains. (image from Vivian et al, 2011).

The involvement of the peptide appears to promote the cross-reactivity of KIR2DL2/3 in a peptide-dependent manner. The presence of alanine at positions P7 and P8 of the peptide promoted cross-reactivity between KIR2DL2 and HLA-Cw5 (Sim et al., 2017). The reduced involvement of presented peptides in KIR2DL1 avidity reduces overall cross-reactivity, though some C1 cross-reactivity is displayed with peptides

containing arginine at P7 (Sim et al., 2017). The highly analogous structure of arginine and lysine, differing only in a propylamine present in the latter and absent in the former, means arginine at P7 of the peptide may replace the functionality of Lysine at position 80 of the HLA-Cw molecule in cases of cross-reactivity (Sim et al., 2017). The dependence of KIR cross-reactivity upon peptides may provide a mechanism by which C1 or C2 homozygous individuals can license the required KIR receptors when viral peptides are presented (Sim et al., 2017). Unfortunately, involvement of viral peptides can also facilitate viral evasion, suppressing NK cell cytotoxic action via inhibitory KIR stimulation in individuals positive for the cognate HLA class I ligand (van Teijlingen et al., 2014; Hölzemer et al., 2015; Schafer et al., 2015; Lunemann et al., 2016; Wauquier et al., 2019).

Similar to the KIR2D inhibitory KIR receptors, the P8 presented peptide residue influences the specificity of KIR3DL1 for HLA class I. Serine, phenylalanine, histidine, and arginine at P8 all facilitate KIR3DL1 binding, while alanine, glutamic acid, and leucine at P8 inhibit KIR3DL1 binding (Vivian et al., 2011).

Polymorphisms within HLA class I molecule have also been identified as influencing KIR3DL1 binding avidity. The gene products of HLA-B*57:01 and B*57:03 differ by only two amino acid residues: positions 114 and 116, both found within the base of the HLA peptide binding cleft (Robinson et al., 2020). These few alterations to the HLA complex are sufficient to present different binding interfaces with KIR3DL1 (Saunders et al., 2020).

1.2.3 Activating KIR Interactions

The relationship between inhibitory KIR and HLA class I proteins is well characterised, but the ligands of activating KIR genes remain largely elusive.

KIR3DS1 has been shown to exhibit affinity for HLA Bw4 and HLA-F (a non-classical HLA class I molecule), and KIR2DS1 binds the C2 epitope (Martin et al., 2002; Stewart et al., 2005; O'Connor et al., 2015; Burian et al., 2016; Dulberger et al., 2017). Evidence has indicated that KIR3DS1 binds to the HLA-B*57:01 protein/peptide complex in a peptide-dependent manner (an HLA allotype which contains the HLA-Bw4 epitope), suggesting that in some cases the presentation of the cognate peptide within the HLA molecule's peptide binding groove is necessary to form a discontinuous epitope for KIR recognition (O'Connor et al., 2015). Investigations into the specificity of KIR2DS2 have also identified specificity for a novel β_2-microglobulin-independent protein, but the identity of this ligand has not yet been ascertained (Thiruchelvam-Kyle et al., 2017). The same study suggests this unidentified ligand may also act as a target for KIR2DL2 and 2DL3.

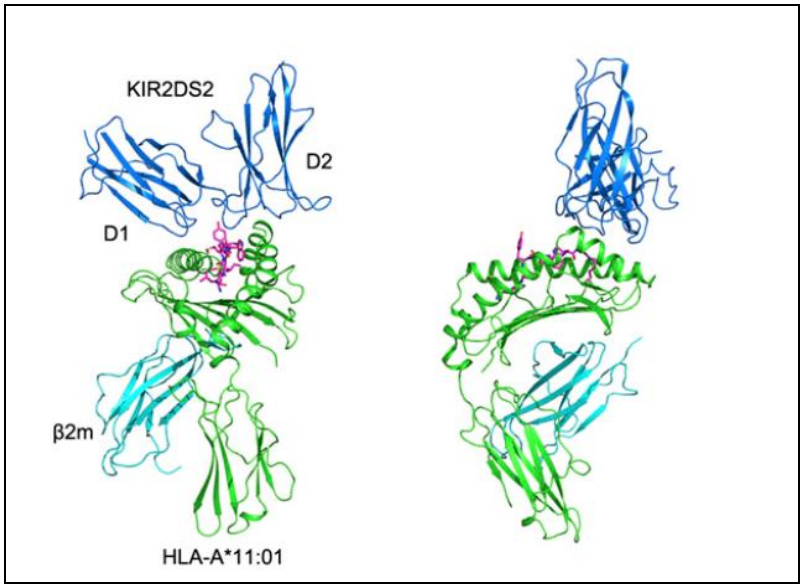


Figure 6 – Structural diagram of interactions between KIR2DS2 and HLA-A11. KIR2DS2 (blue) binds to HLA class I similarly to inhibitory KIR2D glycoproteins, across the proximal end of the HLA peptide binding groove (green, with cyan β_2-microglobulin), and across the peptide itself (magenta) (image from Liu et al, 2014)

Similar to inhibitory KIR, the binding of activating KIR receptors is also dependent upon peptide presented by HLA class I. The P8 residue of the peptide presented by HLA-Cw5 influences KIR2DS4, with tryptophan at this position sufficient for potent activation of even unlicensed NK cells (Sim et al., 2019). Tryptophan at P8 is found in <1% of self peptides presented by HLA-Cw5, but this characteristic is also found in RecA, a protein essential for the genetic regulation of all free-living bacteria, (Bell and Kowalczykowski, 2016; Sim et al., 2019). KIR2DS4 is therefore specific for 'rare self', with the frequency of this peptide characteristic increasing many-fold at times of infection (Sim et al., 2019). The specificity of activating KIR for peptides may also be leveraged in viral evasion, with hyper-stimulation of NK cells via KIR2DS1 and 2DS3 leading to the deletion of the NK cell compartment (Wauquier et al., 2010). Similarly, the P8 residue of the peptide is significant in KIR2DS2 interactions, with charged P8 residues reducing avidity of the KIR (Figure 6) (Liu et al., 2014).

KIR2DL3*0020103	cA01~tB02
Key:	**Key:**
KIR – Acronym 2D – 2 Ig-like domains L – long cytoplasmic tail] 3 – third 2DL protein identified * - separator 002 – encodes member of the second series of KIR2DL3 proteins. differs from other KIR2DL3*002 genes by non-synonymous DNA substitution 01 – differs from other KIR2DL3*002 genes by synonymous mutation 03 – differs from the other KIR2DL3*00201 alleles within a non-coding region	c/t – Centromeric/Telomeric Region of KIR gene cluster A/B – KIR A/B Haplotype characteristics Two digits – Label, denoting the specific region characteristics according to presence/absence of KIR genes and copy number variation

Figure 7 – Examples of the standardised nomenclature for describing KIR alleles (left) (Marsh et al., 2003), and KIR gene content (right) (Pyo et al., 2010; Vierra-Green et al., 2012; Pyo et al., 2013; Vierra-Green et al., 2016).

1.3 KIR Genomics

The KIR gene cluster on chromosome 19q13.4 is composed of up to thirteen KIR genes that encode inhibitory, activating and immunomodulatory receptors, as well as

two pseudogenes, KIR2DP1 and 3DP1 (Nomenclature described in Figure 1) (Marsh et al., 2003; Gomez-Lozano et al., 2005; Pyo et al., 2010; Vierra-Green et al., 2012; Pyo et al., 2013; Gonzalez-Galarza et al., 2015; Vierra-Green et al., 2016). An additional KIR pseudogene, KIR3DX1 (originally named KIR3DL0), is located separately on chromosome 19, between the leukocyte immunoglobulin-like receptor (LILR) clusters (Sambrook et al., 2006). Gene content is variable within the population, with high diversity observed in both the presence/absence and copy number variation (CNV) of each gene (Uhrberg et al., 1997). KIR genes also exhibit allelic polymorphism, providing multiple levels of intra- and inter-population variation.

Unable to undergo somatic recombination to establish variation in specificity and functional capacity like their adaptive lymphocyte counterparts, NK cells undergo variegated expression of KIR and C-type lectin receptors to constitute diverse cellular sub-populations with different functional characteristics. This is achieved by the selective silencing and expression of individual KIR genes in a stochastic manner, ultimately affecting the NK cell's phenotype, specificity and functional capacity for cytotoxic action (Cichocki et al., 2011; Manser et al., 2015). It is of note that many activating and inhibitory KIR (aKIR and iKIR, respectively) appear to compete for the same ligands (Pende et al., 2019). Competing signals from aKIR and iKIR proteins may provide a refined degree of variance in functional capacity between NK cell subpopulations (Manser et al., 2015).

1.3.1 Genetic Structure

The structure of KIR genes is relatively conserved. Each gene (excluding KIR3DP1) consists of eight (the two domain KIR genes and KIR3DL3) to nine exons (the

remaining three domain KIR genes) (Vilches and Parham, 2002). Exons 1 and 2 constitute the leader sequence, exons 3-5 form the extracellular D0, D1, and D2 domains respectively. Exon 6 forms the stem, connecting the D2 domain to the transmembrane region (exon 7), with exons 8 and 9 translating to the cytoplasmic tail. Type 1 KIR2D genes (KIR2DL1-3 and 2DS1-5) possess a pseudoexon 3, resulting in proteins with only D1 and D2 extracellular domains (Vilches et al., 2000b). Type II KIR2D genes (KIR2DL4 and 2DL5) lack an exon in position 4, resulting in a protein with D0 and D2 extracellular domains (Selvakumar et al., 1996; Vilches et al., 2000a). KIR2DP1 is a type I KIR2D gene, possessing eight exons and a pseudoexon 3. KIR3DL3 lacks a stem region (exon 6), with a remnant of the stem region sequence incorporated into the transmembrane region (exon 7) (Trundley et al., 2006). KIR3DP1 is the shortest of the KIR genes with only exons 1-5, with some KIR3DP1 alleles (such as KIR3DP1*003) presenting a deletion of exon 2 (Gomez-Lozano et al., 2005). KIR3DP1 possesses a sixth exon, but this is derived from an intergenic region and shares no known homology with any other KIR gene, and so has been denoted as exon 6b to avoid confusion.

1.3.2 Gene Content

The KIR gene cluster is one of the most variable regions in size and gene content within the entire genome, owing to non-allelic homologous recombination (NAHR) events resulting in the insertion and deletion of entire KIR genes into/from haplotypes. One feature that is unique to humans is the division of KIR haplotypes into two distinct categories: group A and B haplotypes (Figure 8) (Parham et al., 2012). These groups were originally distinguished by the presence or absence of a 24kb band when digested by HindIII restriction enzyme and analysed by Southern Blot technique (Uhrberg et al., 1997; Wilson et al., 2000; Hsu et al., 2002b; Gonzalez-Galarza et al., 2015).

KIR Diplotype ID	Centromeric							Centromeric/ Telomeric			Telomeric						Individuals	Populations
	KIR3DL3	KIR2DS2	KIR2DL2	KIR2DL3	KIR2DP1	KIR2DL1	KIR3DP1	KIR2DL5	KIR2DS3	KIR2DS5	KIR2DL4	KIR3DL1	KIR3DS1	KIR2DS1	KIR2DS4	KIR3DL2		
A/A1	P	a	a	P	P	P	P	a	a	a	P	P	a	a	P	P	7,540	190
B/x2	P	a	a	P	P	P	P	P	a	P	P	P	P	P	P	P	2,522	178
B/x4	P	P	P	P	P	P	P	a	a	a	P	P	a	a	P	P	2,096	178
B/x5	P	P	P	P	P	P	P	P	P	a	P	P	a	a	P	P	1,536	161
B/x3	P	P	P	P	P	P	P	P	a	P	P	P	P	P	P	P	1,157	167
B/x6	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	899	155
B/x8	P	a	a	P	P	P	P	P	P	a	P	P	P	P	P	P	635	130
B/x7	P	P	P	P	P	P	P	P	P	a	P	P	P	P	P	P	583	134
B/x71	P	P	P	a	P	P	P	P	P	a	P	P	a	a	P	P	443	112
B/x9	P	P	P	P	P	P	P	P	a	P	P	P	a	P	P	P	395	120

Figure 8 – Table illustrating the 10 most commonly observed KIR haplotypes (by number of individuals) from a total of 660 different KIR diplotypes

Data collected from studies covering 191 populations and 24,950 individuals (Gonzalez-Galarza et al., 2015).

Key: *P=Gene present, a=Gene absent; Red=Inhibitory gene, Green=Activating gene, Purple=Pseudogene, Grey=Immunomodulatory gene during placentation of pregnancy.*

With some exceptions, both group A and B haplotypes contain the framework genes KIR2DL4, 3DL2, 3DL3, and pseudogene KIR3DP1 (Figure 8, Figure 9). Group A haplotypes consist of a largely homogeneous set of up to seven genes and a second pseudogene: KIR3DL1, 2DL1, 2DL3, 2DS4, and 2DP1, accompanied by the four framework genes. The number of genes observed within group B haplotypes vary considerably, but are broadly defined by the inclusion of further aKIR and iKIR genes in assorted combinations (Gonzalez-Galarza et al., 2015). Consequently, individuals are often categorised as possessing an A/A diplotype (for A haplotype homozygous) or B/x diplotype (for A/B haplotype heterozygous or B/B haplotype homozygous).

Due to the inclusion of several aKIR genes, group B haplotypes are regarded as the "activating" haplotype.

A hotspot for recombination is positioned in the centre of the KIR gene cluster (between KIR3DP1 and 2DL4), dividing the gene cluster into two regions: the centromeric region (region closest to the centromere of the chromosome) and telomeric region (region closest to the telomere of the chromosome) (Figure 9) (Hsu et al., 2002a; Jiang et al., 2012). The linkage disequilibrium between genes within each region has led to the theory that most common haplotypes are derived from a limited assortment of centromeric and telomeric regions arranged in differing combinations (Pyo et al., 2010; Vierra-Green et al., 2012). As such, it is possible for a haplotype to consist of a combination of a centromeric region with the characteristics of an A haplotype and telomeric region with the characteristics of a B haplotype, or vice versa. These characteristics have inspired the development of haplotype nomenclature described in Figure 7.

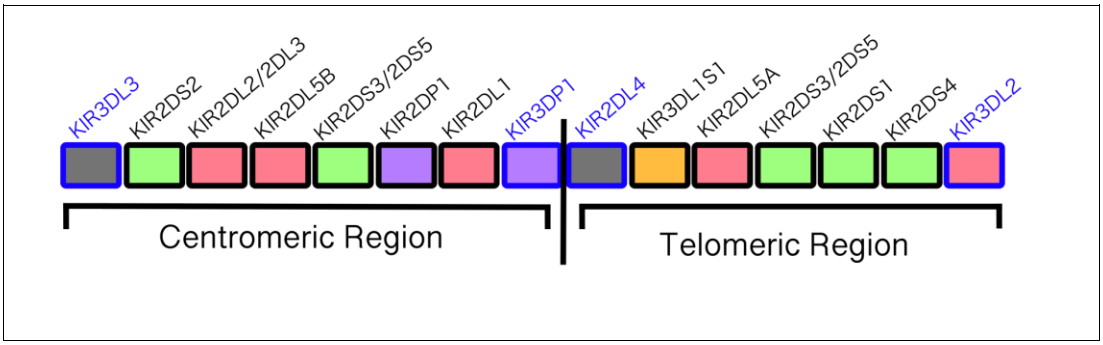
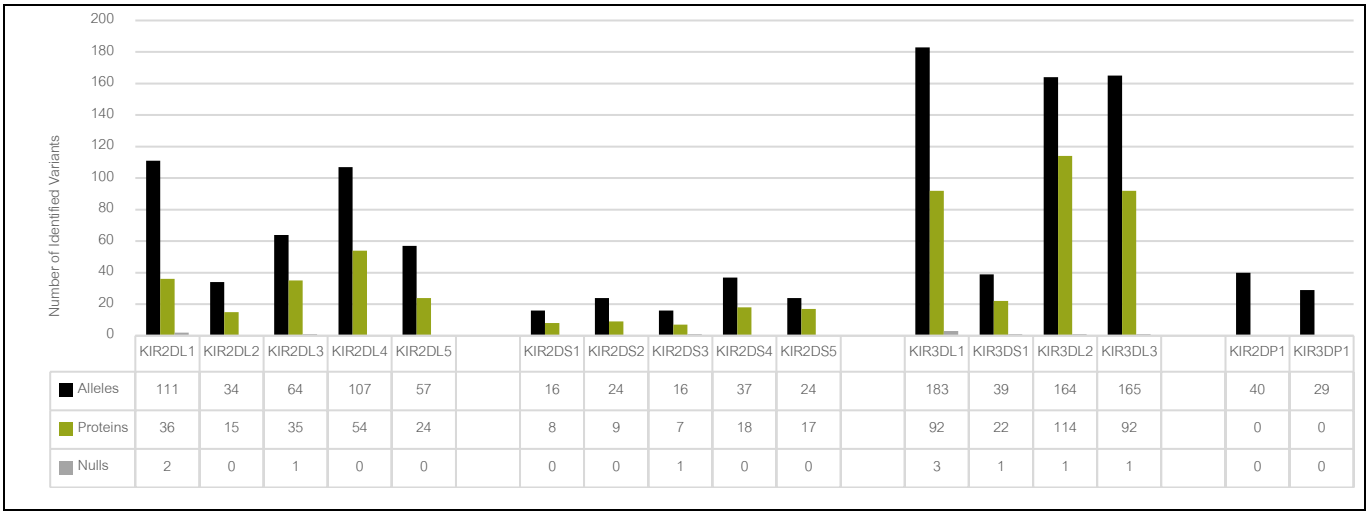


Figure 9 – KIR gene cluster map demonstrating approximate locus and regional location.

Each KIR haplotype will contain a different gene content comprising of the four framework genes accompanied by an assortment of non-framework genes (Hsu et al., 2002a). Hotspot for recombination, separating the centromeric and telomeric region, indicated by vertical line. ***Key:*** *Red=Inhibitory gene, Green=Activating Gene, Purple=Pseudogene, Gold=Inhibitory/Activating gene dependent upon allele present, Grey=Immunomodulatory gene during placentation; Genes in blue border/typeface=Framework genes.*

1.3.3 Allelic Polymorphism

The presence or absence of a gene only provides partial information on KIR genetics and proteomics. KIR genes are highly polymorphic, with 977 alleles identified as of the latest IMGT statistical release in November 2018 (Figure 10) (EMBL-EBI, 2019). This polymorphism appears to have arisen in association with the evolution of gene content variation, with population studies identifying linkage disequilibrium between several KIR gene polymorphism and A or B haplotypes (Hilton et al., 2015b). The variety of defined KIR alleles is rapidly expanding, with each new study of KIR alleles likely to identify novel and previously unpublished alleles. This has led to the suggestion that the degree of polymorphism of KIR in the global population may ultimately equal or exceed that of HLA, currently accepted as the most polymorphic gene system in the human genome (Misra et al., 2018).



	KIR2DL1	KIR2DL2	KIR2DL3	KIR2DL4	KIR2DL5	KIR2DS1	KIR2DS2	KIR2DS3	KIR2DS4	KIR2DS5	KIR3DL1	KIR3DS1	KIR3DL2	KIR3DL3	KIR2DP1	KIR3DP1
Alleles	111	34	64	107	57	16	24	16	37	24	183	39	164	165	40	29
Proteins	36	15	35	54	24	8	9	7	18	17	92	22	114	92	0	0
Nulls	2	0	1	0	0	0	0	1	0	0	3	1	1	1	0	0

Figure 10 – Number of alleles, respective proteins, and null genes identified for each KIR gene as of the latest statistical IMGT release, December 2019 (EMBL-EBI, 2019).

NB: KIR3DL1 and KIR3DS1 have been identified as distinct allele groups of the same gene, KIR3DL1S1, but have been separated in this chart to demonstrate the variation observed within each functional isoform (Marsh et al., 2003).

Allelic polymorphisms can significantly alter the affinity and specificity of KIR proteins for their target epitope. For example, the KIR receptor encoded by KIR2DL1*022 has specificity for the C1 epitope (in contrast to the C2 specificity

observed in a majority of KIR2DL1 receptors) due to a methionine to lysine substitution at position 44 (Hilton et al., 2015a). Similarly, KIR2DL1 allotypes within the centromeric region of haplotype A (CenA) display higher avidity for C2 epitopes than those of haplotype B (CenB). The weaker receptor avidity of CenB KIR2DL1 allotypes are largely determined by four positions within the D2 domain: positions 114, 154, 163, and 182 (Hilton et al., 2015a). In addition, some CenB KIR2DL1 allotypes are further impaired by polymorphisms within the transmembrane domain acting as a key determinant of limited surface expression (Hilton et al., 2015a). Conversely, the allotypes of C1-specific iKIR within CenB haplotypes (predominantly KIR2DL2) exhibit higher avidity for both C1 epitopes than those of CenA haplotypes (KIR2DL3) (Hilton et al., 2015a). The C1-specific iKIR of B haplotypes also displaying a degree of cross-reactivity for C2. These findings demonstrate both the impact of genetic polymorphism upon KIR phenotypical properties and the significant disparity between the activity of KIR proteins encoded by group A and B haplotypes.

Wider phenotypical variation can be influenced by allelic polymorphism. One common allele of KIR2DS4, KIR2DS4*0030101, possesses a gene truncation upstream of the transmembrane domain (Maxwell et al., 2002; Middleton et al., 2007). The phenotypical impact of this mutation is unknown. If expressed, it has been suggested that the gene product may be secreted into the surrounding environment to quench soluble HLA, enhancing NK cell activity (Middleton et al., 2007). Allelic polymorphism can also dictate the broad signalling functionality of the KIR protein, with KIR3DL1 (an iKIR) and 3DS1 (an aKIR) found to be alleles of the same gene, KIR3DL1S1 (Marsh et al., 2003). In spite of this, due to the difference in functionality, KIR3DL1 and 3DS1 continue to be considered separately in many studies.

1.3.4 Copy Number Variation

The same NAHR events that give rise to variation in gene content in KIR haplotypes also result in CNV, presenting multiple copies of the same gene within a single haplotype (Traherne et al., 2010). As with gene content variation, NAHR is predominantly observed in group B haplotypes, with one study finding >80% of B haplotypes displaying evidence of NAHR compared to <1% in A haplotypes in a cohort of US/UK families – further evidence of the conserved nature of A haplotypes (Jiang et al., 2012). This data strengthens the theory that NAHR (and consequently, CNV) is more important to the evolution of the B haplotype than the A haplotype, but also raises questions concerning the evolutionary value of duplicating genes.

Possessing multiple copies of a KIR gene increases the likelihood that gene will be expressed, increasing the prevalence of NK cell subpopulations presenting the respective receptor (Béziat et al., 2013; Gamazon and Stranger, 2015). However, CNV does not appear to increase the surface density of the KIR receptor, nor enhance the functional responses of the NK cell. These findings suggest CNV may hold clinical significance in the context of NK cell subpopulation composition within an individual, but not at the level of an individual NK cell (Béziat et al., 2013).

The NAHR that governs CNV of KIR appears to be spatially restricted, with an inconsistent degree of CNV between KIR genes. The genes at the extremities of the cluster, such as KIR3DL2, 3DL3, 2DS2, 2DS1, and 2DS4, present limited duplication in comparison with the high frequency of duplication observed in KIR3DL1S1 at the centre of the cluster (Jiang et al., 2012; Vendelbosch et al., 2013). The tight restriction of NAHR within the limits of the KIR gene complex suggests this mechanism is of

central importance to the genetic evolution of the KIR gene cluster, and may explain why the primary hotspot for recombination is located at the centre of the gene cluster.

1.3.5 Expression of KIR genes

The expression of KIR genes is clonally distributed in a variegated manner as a result of stochastic expression selectively silencing KIR genes via methylation, so that no single NK cell expresses all KIR genes contained within the genome (Santourlidis et al., 2002; Manser et al., 2015). KIR genes are hypo-methylated by default, suggesting other epigenetic mechanisms may silence KIR genes in immature NK cells (Chan et al., 2003). Each KIR gene has two promoters: a distal unidirectional promoter and a proximal bidirectional promoter. By simultaneously transcribing from the distal sense strand and the proximal antisense strand, it is possible to produce dsRNA, which has been shown to epigenetically mediate methylation of other genes (Davies et al., 2007). The one exception to this epigenetic mediation is KIR2DL4, which is constitutively expressed (Santourlidis et al., 2002; Trompeter et al., 2005). The promoter of KIR2DL4 does exhibit small variances in structure compared to that of clonally distributed KIR genes, though this does not appear to be sufficient to solely explain the large difference in expression characteristics (Trompeter et al., 2005).

With variegated stochastic expression, no KIR phenotype represents greater than 7% of the overall NK cell population in the peripheral circulation (Horowitz et al., 2013; Manser et al., 2015). The proportion of phenotypes varies between individuals, with evidence of genetic, epigenetic and environmental influences. In all cases, the largest sub-population of NK cells presents no KIR on its cell surface (41-72% of NK cells), with expression of NKG2A providing the primary NK cell immunosurveillance activity (Horowitz et al., 2013; Manser et al., 2015). Of NK cells with KIR expression,

most express between one and three iKIR, with suggestions that cells with fewer iKIR have a larger presence in the peripheral circulation to facilitate rapid expansion to therapeutic levels for an effective immune response when required in times of infection (Manser et al., 2015). NK cells with several KIR expressed provide a more specific immune defence, which requires a longer period of time to constitute the necessary therapeutic levels due to lower constitutive numbers, acting in a similar time frame to that of the adaptive immune system (Manser et al., 2015). This observation strengthens the notion of NK cells acting as a bridge between the innate and adaptive immune systems.

Model	Description	Most Advantageous Donor (as Described by Model)
KIR Receptor-Ligand Mismatch Model (RLM) (Leung et al., 2004; Park et al., 2015b; Faridi et al., 2016)	A KIR mismatch is described as the donor possessing inhibitory KIR for which the recipient lacks the ligand (i.e. HLA Class I epitope).	RLM KIR mismatched donor
KIR Ligand-Ligand Mismatching Model (KLM) (Ruggeri et al., 1999)	A KIR mismatch is described as the donor possessing a KIR ligand (i.e. HLA Class I epitope) which is absent in the patient.	KLM KIR mismatched donor (Thought to be an inferior predictor of transplant outcome compared to RLM)
Missing Licensing Proof Model (Nowak et al., 2014; Nowak et al., 2015)	A 'Missing Licensing Proof' is described as a lack of a cognate activating KIR ligand in the patient for which the donor has licensed NK cells (i.e. donor possesses inhibitory KIR receptor and cognate ligand, patient lacks cognate ligand).	Donor without missing licensing proof
KIR B-Content Scoring Model (Cooley et al., 2009; Cooley et al., 2010)	Transplants from donors possessing a KIR B haplotype present improved outcomes post-transplant due to the presence of stimulatory KIR genes.	Donor possessing KIR *CenB/B* haplotypes
KIR Matching Model (Faridi et al., 2016; Sahin et al., 2018)	The KIR diplotype between patient and donor is matched in a HLA matched sibling donor setting. Compared either as a complete diplotype, or with iKIR and aKIR matching assessed separately.	KIR matched sibling (if a complete match is not possible, iKIR-matching is preferred to aKIR-matching)
Missing KIR Ligand Model in Autologous HPCT (Leung et al., 2007)	In autologous transplantation, a KIR mismatch is described as the patient possessing an inhibitory KIR for which they do not possess the ligand (i.e. HLA Class I epitope).	N/A (Only applicable to autologous transplantation)

Table 1 – Summary of several models for recipient/donor KIR assessment in HPCT.

1.4 KIR & Haematopoietic Progenitor Cell Transplantation

1.4.1 KIR Analysis in Donor Selection

The immunoregulatory role of NK cells presents an effective clinical utility in HPCT. HLA matching remains the primary tool for selecting optimal donors, with KIR genes providing a potential supplementary selection tool for donors of equal HLA match, age and CMV serostatus match in accordance with existing recommendations (Howard et al., 2015; Shaw et al., 2017; Mayor et al., 2019). With the patient and donor possessing different KIR genes and ligands, patient/donor pre-transplant assessments for selection of the optimal donor present a complex set of interactions to study in the context of HPCT outcomes and the patient's diagnosis. As KIR and HLA genes are located within different chromosomes, even siblings who are HLA matched only present a 25% probability of also being KIR matched, due to the Mendelian model of genetic inheritance. This opens the opportunity for assessing KIR even in cases of potential HLA matched sibling donors. Several competing models in assessing the impact of KIR compatibility on HPCT outcomes have been devised, but a majority share a primary aim of increasing donor NK cell reactivity towards patient cells to preferentially target malignancy (Table 1).

The wealth of data concerning the influence of KIR genes in HPCT outcomes is confounding in its diversity of findings and lack of a universally applicable model that works successfully in all transplant centres. Conflicting findings are numerous, with few commonalities in treatment, patient cohorts, and study protocols between centres. The lack of standardisation across study protocols hinders the ability to optimise clinical models with the information available, and obfuscates the relevance of any direct comparison between studies. Areas of variability include the definition of KIR

compatibility, the large number of variable factors associated with HPCT programmes (such as diagnosis, disease stage, other donor selection criteria, donation unit pre-treatment, and conditioning regimes), and the small sample sizes involved in many studies. Due to the lack of a universally applicable KIR assessment model, it is good practice for any transplant centre to assess the clinical effectiveness of their KIR assessment protocol using their local cohort of patients to ensure the respective protocol is of clinical value.

The contradictory transplant outcomes between transplant centres in the application of KIR assessment models is partially due to variance in the wider transplantation protocols used in different centres (Cooley et al., 2010; Sobecks et al., 2015). This divergence of outcomes in response to conditioning has been confirmed by direct comparative analysis between the transplantation regimes of RIC and myeloablative conditioning (MAC) (Weisdorf et al., 2020). In addition to chemotherapy, NK cells have demonstrated sensitivity to radiotherapy, with irradiation enhancing the cytotoxic activity of NK cells in animal models (Canter et al., 2017). This mounting evidence suggests that KIR assessment models may need to be utilised in the context of the intended transplantation protocol to maximise the benefits in post-transplant outcomes. Further comparative studies are required to fully understand the influence of conditioning regimes upon NK cells and KIR assessment models.

The sensitivity of KIR models to conditioning regimes may be attributable to NK cell reconstitution post-transplant occurring earlier than that of other lymphocytes. NK cells reconstitute by two weeks post-transplant, representing the majority of lymphocytes until at least one-month post-transplant (Chan et al., 2018). Early reconstitution of NK cells is associated with improved post-transplant outcomes

through reduced risk of relapse and GvHD, and improved overall survival (Pical-Izard et al., 2015; Minculescu et al., 2016; Chan et al., 2018). Despite early reconstitution, NK cells do not reach end-stage maturity until at least six months post-transplant, with the development process possibly suppressed by calcineurin inhibitors used in GvHD prophylaxis protocols (Wang et al., 2007; Pical-Izard et al., 2015). Expression of KIR genes appears to be largely unaffected by this stymied development, with KIR expression observed at approximately one-month post-transplant, though expression of KIR2DL1/S1 appears reduced (Pical-Izard et al., 2015; Chan et al., 2018).

1.4.2 Gene Content Models for Donor Selection

HPC donors possessing KIR B/x diplotypes are widely accepted to present the optimal option for HPCT when choosing between donors who are of an equal HLA match with the patient (Cooley et al., 2009; Cooley et al., 2010; Symons et al., 2010; Cooley et al., 2014; Oevermann et al., 2014). *CenB* regions in particular have been noted to provide a significant protective effect in AML patients (Cooley et al., 2010; Cooley et al., 2014; Zhou et al., 2014; Bao et al., 2015). The beneficial effects of KIR B/B donors are greater than double those observed when using KIR A/B diplotype donors, indicating that A haplotypes may also have a causative detrimental impact upon HPCT outcomes (Cooley et al., 2010).

The KIR B Content Scoring model is a commonly used HPCT donor selection algorithm derived from these findings, prioritising B haplotype donors due to their activating characteristics (Table 2) (Cooley et al., 2009; Cooley et al., 2010; Cooley et al., 2014). This algorithm categorises potential donors as ‘Neutral’, ‘Better’, or ‘Best’, with these categories representing approximately 70%, 20% and 10% of the international donor registry pool respectively (Weisdorf et al., 2019). The B content ranking system places Cen-B/B donors as the optimal option, as a high B-content in

the donor is likely to result in increased NK cell activation. For ease of interpretation, a 'Donor KIR B-content Group Calculator' has been provided for free by the European Bioinformatics Institute (EMBL-EBI), allowing transplant centres to accurately categorise their prospective donor's KIR content (Cooley et al., 2010; EMBL-EBI, 2017).

KIR B-content Category	Definition Criteria
Neutral	KIR B-content score ≤1 (KIR2DL3 present, KIR2DS2 and/or KIR2DL2 absent, KIR3DL1 and KIR2DS4 present)
Better	KIR B-content score ≥2 Cen-A/x, Tel-B/x (KIR2DL3 present, KIR3DS1 and/or KIR2DS1 present)
Best	KIR B-content score ≥2 Cen-B/B, Tel-x/x (KIR2DL3 absent, KIR2DS2 and/or KIR2DL2 present)

Table 2 – Definition of the KIR B-content categories used to rank potential HPCT donors (Cooley et al., 2009; Cooley et al., 2010).

The KIR B-content scoring system is based upon assessing the KIR A/B content for the centromeric and telomeric region of each haplotype (Cen-X/X, Tel-X/X). Consequently, four regions are evaluated, with each region exhibiting B content characteristics providing a score of 1. The final score produced is between 0 and 4. Additional weighting is provided for donors homozygous for centromeric KIR B, due to the greater influence of the centromeric region KIR genes in post-transplant outcome. For example, KIR Cen-B/B, Tel-A/A would score 2 for KIR B-content, and would be categorised as 'Best' due to Cen-B/B.

The Receptor-Ligand Mismatching (RLM) model is a competing model that aims to mismatch donor iKIR and recipient iKIR ligand to reduce donor NK cell inhibition, and consequently increase NK cell cytotoxic activity (Leung et al., 2004; Park et al., 2015b; Faridi et al., 2016). This model describes a KIR mismatch as the absence of a KIR ligand in the patient that is recognised by the donor's KIR repertoire, taking advantage of the 'missing self' model to drive NK cell activation. KIR mismatching using the RLM demonstrates a trend towards improved overall survival and disease free survival, as well as decreasing the risk of relapse (Leung et al., 2004; Park et al., 2015b). As a precautionary measure, the RLM includes a recommendation that the donor NK cells exhibit at least one inhibitory KIR specific for a recipient ligand to

maintain NK cell immunoregulatory function, and minimise the risk of NK cell autoimmunity post-transplant, though no case of such autoimmunity has yet been observed (Leung et al., 2004; Gianchecchi et al., 2018). The RLM has also been found to be clinically significant in cases of autologous HPCT, with patients possessing KIR for which they do not possess the ligand experiencing improved rates of disease-free survival following transplant(Leung et al., 2007). As autologous transplantation by its nature does not involve donor selection, this observation is of limited clinical utility.

The KIR Ligand-Ligand Model (KLM) functions similarly to the RLM, but defines a KIR mismatch as the absence of a KIR ligand in the recipient that is present within the donor ligand repertoire (a graft versus host (GvH) mismatch) (Ruggeri et al., 1999). As with the RLM, KIR mismatches in the KLM offer an improved overall survival rate (Wu et al., 2015). This data is further supported by the finding that host versus graft (HvG) KIR ligand mismatches result in a higher risk of relapse (HR=10.7, p=0.002) and lower 2-year disease free survival (HR=3.4, p=0.025) compared to GvH mismatches (Yahng et al., 2016). Not all laboratories have access to KIR genotyping techniques, and so the KLM may provide value to these centres. Unfortunately, mismatching KIR ligands necessitates the mismatching of HLA genes – one of the most significant factors in post-transplant outcomes (Mayor et al., 2019). The detrimental effect of HLA mismatching must be considered when using a KIR ligand mismatching model. There are few comparative analyses to draw conclusions on comparative clinical relevance between the relatively similar KLM and RLM models, and so more research is required to ascertain which model is more beneficial to patient care (Leung et al., 2004).

Further research has focussed on improving the clinical relevance of the RLM model influenced by considering basic scientific principles. A three level Missing Licensing Proof model has been constructed that enhances the RLM model to consider the effect of NK cell licensing on donor selection (Nowak et al., 2014; Nowak et al., 2015). Both the KIR and HLA profiles of the donor are assessed, with a presumption that the presence of an KIR gene coupled with the presence of the cognate KIR ligand gene in the donor will result in the donor possessing licensed NK cells expressing that iKIR gene. Conversely, if the donor possesses the iKIR, but not its ligand, the respective donor NK cell subpopulation will not fulfil licensing. The HLA profile of the patient is then assessed for KIR ligands to ascertain whether the patient possesses the KIR ligands for the donor's licensed NK cells. This model has been applied in the context of iKIR and aKIR genes. These studies found that HPCT recipients who possessed the cognate iKIR ligands for the donor's licensed NK cells experienced superior overall survival in leukaemia patients compared, suggesting the presence of these ligands in the recipient promote immunosurveillance of malignancy post-transplant (Nowak et al., 2014). A similar beneficial effect was observed in the case of aKIR, with improved progression-free survival and reduced rate of disease progression associated with aKIR licensing proof (Nowak et al., 2015).

The findings of the iKIR missing licensing proof models appear antithetical to the principles of the RLM, where absence of the cognate ligand in the patient was found to be beneficial. Nowak et al argue that these opposing findings are due to the lack of consideration for the influence of NK cell licensing in many 'missing self' models, with this additional layer of analysis providing details of the donor NK cells' ability to provide a fully licensed anti-malignancy effect (Nowak et al., 2014; Nowak et al., 2015). Without fulfilling licensing of NK cells via their KIR, the lymphocyte will be

largely hyporesponsive to stimulus (Tu et al., 2016; Boudreau and Hsu, 2018). The proportion of HPCT recipients with this detrimental missing licensing proof within these studies was small (<5%), and so a larger study is required to verify these results. This is likely an indication that relatively few patients may benefit from implementation of the missing licensing proof model.

In conflict with the majority of studies assessing the influence of KIR mismatching, some groups have investigated matching KIR diplotypes between donor and recipient, either as a complete diplotype or by assessing iKIR and aKIR genes separately (Faridi et al., 2016; Sahin et al., 2018). KIR matching has been found to reduce risk of chronic graft versus host disease (cGvHD), with iKIR matching presenting a stronger influence in outcomes over aKIR(Faridi et al., 2016; Sahin et al., 2018). No correlation were found with relapse rates, acute graft versus host disease (aGvHD) or survival, but reduced relapse and improved relapse-free survival was observed when the RLM model was applied to the KIR-matched group (Faridi et al., 2016). Unfortunately, applying HLA matching, KIR matching and the RLM model in parallel is only applicable to patients who present a KIR/Ligand mismatch within their own genotype, and greatly reduces the optimal potential pool of donors who fit these criteria when assessed in parallel with other established criteria (such as CMV serostatus and age of donor).

As the presence of ligands is essential to the licensing process of NK cells, recipient KIR ligand diversity presents further context for KIR gene content assessment models. C1/C1 recipients present improved overall survival post-HPCT and reduced risk of relapse compared to C1/C2 and recipients, but this is offset by an increased risk of grade III/IV aGvHD (Park et al., 2015a; Sobecks et al., 2015; Neuchel et al., 2017;

Arima et al., 2018). The detrimental effect of lacking the C1 epitope (approximately 15% of the European population) is well characterised, with C2/C2 recipients experiencing reduced overall survival and disease-free survival, and increased incidence of relapse compared to C1 positive recipients (Sobecks et al., 2015; Neuchel et al., 2017). iKIR specific for C1 are the first NK cell effector receptors to be expressed during post-transplant immune reconstitution, and so early phase NK cells in C2/C2 patients fail to undergo licensing, greatly reducing the functional capacity of NK cells in the initial months post-transplant (Miller and McCullar, 2001; Fischer et al., 2007).

These findings have raised concerns that C2/C2 patients represent an underserved demographic, who could be aided by improved donor selection protocols. It has been suggested that donor selection based on donor KIR gene content could have further beneficial and detrimental effects on the transplant outcomes of C2/C2 patients. KIR2DS1 positive donors may offer reduced relapse incidence for C2/C2 patients, but also increase the risk of transplant-related mortality (TRM) (Sobecks et al., 2015; Neuchel et al., 2017). KIR2DS2 positive donors may also be beneficial for C2/C2 patients receiving a 9/10 HLA matched graft, reducing TRM without affecting relapse rates (Neuchel et al., 2017). Unfortunately, established donor selection algorithms, such as the B content scoring model, may offer no benefit for C2/C2 patients (Cooley et al., 2014; Faridi et al., 2016).

1.4.3 Limitations of Assessing Gene Content in HPCT

The clinical models for selecting HPCT donors described thus far have presented a convincing argument for clinical consideration of KIR genes in HPCT. However, there remains a large knowledge gap between our understanding of the KIR

genetics/proteomics and the structure of clinical models, making it difficult to explain precisely why and how these models work. Closing this gap requires further scrutiny and advancement in our understanding of both the basic science of KIR and the clinical outcomes of HPCT.

The gene content models described largely neglect to assess the true degree of genetic variation within the KIR gene system. The aKIR content of B haplotypes varies widely, from between two and six activating receptor genes, but these haplotypes are often treated within algorithms as a single homogenous group. In addition, studies into NK cell activity have provided evidence for the functional variation between both different KIR genes and different alleles of the same KIR gene (Pando et al., 2003; Boudreau et al., 2016). Assessment of the full extent of genetic variation could be of clinical benefit, further stratifying potential HPCT donors in the context of their KIR genotype. Further analysis of KIR gene content and polymorphism is required to ascertain the value of fully assessing the complexities of KIR genetics, and how these factors may be weighted for optimal clinical utility.

A common theme in the described clinical models is the objective of increasing the likelihood of activating donor NK cells. For example, the RLM aims to ensure maximal NK cell activation by minimising NK cell inhibition, while the B-content scoring model ensures maximal NK cell activation by selecting donors with superior aKIR gene content, regardless of the patient's HLA profile. It may be possible to unify these two concepts into a single model, but it is likely that a properly conducted prospective double blinded multi-centre clinical study of a large patient cohort is required to assess the true clinical significance of a more complex model.

Finally, a pragmatic strategy must be employed when considering the practical utility of more complex models. Many patients do not have access to a wide pool of potential donors to select from, and so an overly complex algorithm may not be effective or efficient for donor selection. Even when considering the large pool of donors available from international bone marrow donor registries, there are then cost implications in ordering and testing samples from many prospective donors to identify the optimal donor. For example, to identify a better or best category unrelated donor, in accordance with the B Content Scoring model, it is recommended to order at least four donors (if selected from a pool of donors with no KIR genotyping available) (Weisdorf et al., 2019). A more complex model would likely necessitate ordering further donors. This fiscal burden on clinical laboratories could be reduced by donor registries providing KIR genotyping of donors at the point of registration, but there is currently little impetus to take on this additional testing. With the growth of research into KIR genetics, there are initial indications of some registries taking on this typing, with DKMS currently undergoing KIR genotyping of their patient population, with a projection to provide allele-level KIR genotyping data on 5 million donors by 2021 (Wagner et al., 2018).

1.4.4 Assessing Allelic Polymorphism in HPCT

Investigation into the functional heterogeneity of KIR encoded by different alleles of a single KIR gene has encouraged the development of research assessing the influence of allelic polymorphism in HPCT. At its most basic level, polymorphisms can be used to identify potentially non-functional or non-expressed genes. For example, KIR3DL1 proteins can be stratified by the presence of serine or leucine in position 86 within the D0 domain. A majority of allotypes contain serine in this position, resulting in surface expression of the KIR3DL1 protein, but eight series of KIR3DL1 proteins contain

leucine, disrupting protein folding and blocking the protein's release from the endoplasmic reticulum (Pando et al., 2003; Robinson et al., 2015). One study found ~12.2% of HPCT donors (from a predominantly European Caucasian cohort) possessed a KIR3DL1 allotype that was retained intracellularly(Alicata et al., 2016). It has been suggested that intracellular retention of KIR may aid NK cell licensing and/or ensure minimal inhibition of NK cell activity (Taner et al., 2011). Other studies have assessed the influence of KIR2DS4*030101 in donors, though data is conflicting (Wu et al., 2016; Burek Kamenaric et al., 2017).

Initial assessments of polymorphism have measured the influence of single KIR genes upon post-transplant outcomes, categorising transplants on the basis of selective typing of single nucleotide polymorphisms (SNPs) known to be influential in the phenotypical characteristics of the expressed receptor, unifying the basic scientific principles of KIR genetics and proteomics. One of the first candidate KIR genes to be considered in influencing post-transplant outcomes was KIR2DL1. Dimorphism at position 245 (within exon 7, encoding the transmembrane domain) significantly alters the signalling strength of the expressed protein: KIR receptors with arginine at this position (R^{245}) recruit more signalling complexes than receptors with cysteine at the same position (C^{245}) (Bari et al., 2013). HPCT involving donors positive for KIR2DL1-R^{245} (homozygous or heterozygous) presented superior post-transplant outcomes than recipients negative for this allotype, with improved overall survival and progression-free survival.

Harnessing the relationship between KIR proteomic and genomics has been a recent area of focus in investigating KIR allelic polymorphism. Allelic polymorphism of KIR genes can result in differences in the prime phenotypical characteristics of a

receptor: binding affinity, surface density, and/or overall signalling strength (Boudreau et al., 2016). Polymorphism of KIR3DL1 results in alleles with high (KIR3DL1-H, such as KIR3DL1*001, 002), low (KIR3DL1-L, such as KIR3DL1*005, 007), or null (KIR3DL1-null, such as KIR3DL1*004) expression levels (Boudreau et al., 2016). Polymorphisms within HLA-Bw4 appear to display a relationship with KIR3DL1 avidity, with KIR3DL1-H alleles presenting high avidity for HLA-Bw4 ligands presenting isoleucine at position 80 (HLABw4-80I, such as HLA-B57), and KIR3DL1-L alleles presenting high avidity for HLA-Bw4 ligands with threonine at position 80 (HLA-Bw4-80T, such as HLA-B27) (Boudreau et al., 2016). Position 47 of KIR3DL1, a characteristic that displays some correlation with KIR3DL1 expression, also influences HLA-Bw4 avidity. KIR3DL1 alleles encoding valine at this position (such as KIR3DL1*002 and 015) confer stronger avidity for HLA-B*57:01, with observed protection against HIV (Martin et al., 2018).

The relationship between KIR3DL1 and HLA-Bw4 has important implications for the NK cell immunological response. Strong avidity KIR3DL1/HLA-Bw4 pairs have been suggested to elicit a strong NK cell response when the inhibitory signalling is lost (Martin et al., 2007). In HIV infection, individuals possessing KIR3DL1-H and HLA-Bw4-80I exhibit strong protection against HIV progression, with a weaker protective effect observed in individuals possessing KIR3DL1-L and HLA-Bw4-80T (Martin et al., 2007). A similar effect has been observed in individuals possessing KIR3DS1 and HLA-Bw4-80I (Martin et al., 2002). In HPCT treating AML, transplants with strong inhibiting combinations of donor KIR3DL1 with recipient HLA-Bw4 (KIR3DL1-H+Bw4-80I or KIR3DL1-L+Bw4-80T) presented an increased risk of relapse and reduced overall survival (Boudreau et al., 2017). Avoiding donors

possessing strong inhibiting KIR3DL1 for the patient's HLA class I may provide an algorithm for improving HPCT outcomes in AML (Boudreau et al., 2017).

As discussed in section 1.2.1, interactions between KIR3DL1 and HLA class I are highly complex, and so the described effects of KIR and ligands are likely a simplification of the true interactions. For example, not all individuals possessing the optimal KIR3DL1/HLA-Bw4 characteristics experience protection against HIV progression (Martin et al., 2007). Further work is required to understand the full implications of KIR/HLA interactions and how this can be used to optimise clinical outcomes in transplantation and virology.

1.4.5 Mechanisms of Action: Direct or Indirect Activity?

The exact mechanism of action for how NK cells influence post-transplant outcomes remains unclear, but has important implications for how KIR may be assessed in HPCT donor selection. For example, the potent graft versus leukaemia (GvL) effects observed in the initial months immediately post-HPCT drastically diminish in the long term (Leung et al., 2004). It has been suggested that donor subsets of NK cells may only express a single KIR in the initial 3 months following transplantation as part of the receptor acquisition process. Understanding the NK cell population's place in a post-transplant immune system is vital for understanding how this activity can be enhanced for improved post-transplant outcomes. Transplant centres are demonstrating conflicting results when applying a standardised model of KIR assessment to their HPCT donor selection programmes, but this data is rarely published. Understanding the mechanisms of action could help us to understand why these conflicts in findings arise, and would aid the refinement of KIR assessment models so that they provide universal benefits to patients of all transplant centres.

While studies have focussed on the cytotoxic action of NK cells, these lymphocytes may also apply indirect effects via interactions with the adaptive immune system. A recent study into the control of viral load within infected cells by NK cells found that iKIR exerted influence upon the $CD8^+$ T cell response (Boelen et al., 2018). This study demonstrated that HLA-B57 provides a protective quality, reducing the viral load in this patient cohort, and HLA-B35 presents a detrimental effect, with these individuals more susceptible to high viral loads. iKIR was found to exacerbate this effect, in both a beneficial capacity (in association with HLA-B57) and in a detrimental capacity (in association with HLA-B35), by extending the lifespan of $CD8^+$ T cells (Boelen et al., 2018). The iKIR on the surface of the T cell was found to have a direct effect on the T cell, but iKIR on NK cells may also exert an indirect influence.

It is not known whether this finding in viral immune defence has any implications for the transplantation setting. NK cells are the first lymphocyte to reconstitute in the recipient's immune system post-HPCT, considerably earlier than both T cells and B cells (Nguyen et al., 2005). It's possible that NK cells could establish the post-transplant immunological environment for the adaptive immune system, potentially providing opportunity for therapeutic manipulation to enhance a GvL and/or anti-CMV effect. It is also possible that KIR on the surface of T cells may modulate their immune response for similar benefits. More research is required to investigate the interactions between NK cells and the wider immune system, especially in the context of transplantation.

1.5 KIR Genotyping

1.5.1 Current Techniques for KIR Genotyping

Clinical utilisation of KIR genotyping within the field of clinical H&I in the UK has been largely limited to defining gene content (the presence or absence of a gene). This can be achieved using relatively simple techniques, such as PCR-SSO technology or real-time PCR (qPCR) kits. This is sufficient for current gene content assessment models, but does not define KIR genes at an allelic level, and so cannot assess polymorphic variation for further development of new assessment algorithms. Though Sanger sequencing of KIR is possible, it is highly labour intensive to isolate the individual KIR genes for sequencing in separate reactions, and so this technique is not widely used in a clinical H&I setting (Hou et al., 2012). NGS of KIR could greatly improve the ease at which KIR genes are sequenced, providing data for the development of novel KIR assessment algorithms.

1.5.2 Next Generation Sequencing

NGS is one of the latest developments in genetic sequencing techniques, providing many advantages over Sanger sequencing: higher resolution results, at a higher throughput, and potentially at a lower cost (Nelson et al., 2015). The leading platforms for this technology, the Ion Torrent (Thermo Fisher Scientific) and MiSeq (Illumina) systems, differ greatly in their methodologies but share similar scientific principles of reading a sequence by detecting the incorporation of nucleotides into the DNA sequence. In addition, the Pacific Biosciences (PacBio) platform offers Third Generation Sequencing (TGS), facilitating long read sequencing to resolve cys/trans ambiguities and define the gene content of each haplotype. Table 3 summarises the advantages and disadvantages of the available technologies.

Amplicon sequencing (also known as short-range sequencing) is already widely used in clinical H&I laboratories for sequencing of HLA genes, and has been shown to be effective in sequencing KIR (Wagner et al., 2018). The gene of interest is amplified via PCR, followed by amplicon purification and fragmentation into ~150-350bp long sequences. Indices are then incorporated at the peripheries of each amplicon, enabling sample identification, and libraries purified once more. Sample libraries are pooled prior to massive parallel sequencing. During analysis, automated software 'phases' (i.e. joins together homologous overlapping amplicon sequences) the short amplicon reads into a single contiguous sequence by comparing the sequences of each read. Amplicon sequencing typically generates a high yield of shorter read length amplicons (<1kb) for sequencing, and is generally used for exon sequencing only (Carapito et al., 2016). This simplifies library preparation and provides a higher yield of PCR product for a high read depth, but the generation of shorter read lengths requires good quality phasing to align sequences with reference material, and the fidelity of DNA polymerase can reduce the quality of read data (Sims et al., 2014; Carapito et al., 2016). Consequently, the high read depth must provide sufficient confidence to exclude artefacts that may arise due to issues of fidelity in DNA synthesis, and the phasing can be insufficient for resolving all cys/trans ambiguities. Any regions with a high number of repeating motifs are also difficult to sequence due to the inability to effectively phase amplicons without unique sequence motifs to differentiate amplicons.

Technology	Advantages	Disadvantages
Amplicon-Based Short Read (ABSR) NGS	Cheapest form of NGS	Susceptible to allele imbalance PCR artifacts possible
Hybridisation-Based Target-Enrichment (HBTE) NGS	Can use a very small amount of DNA (50ng) Consistent allele balance Can measure copy number variation (CNV) Reduced 'hands on' workload	More expensive than ABSR NGS
Third Generation Sequencing (TGS)	Can be used to accurately define haplotypes Can define CNV and assign to haplotypes Very high throughput Long read eliminates phasing issues	Currently most expensive form of sequencing Prone to transcription errors and PCR artifacts due to long read Low read depth due to long read

Table 3 – The advantages and disadvantages of the available next generation sequencing (NGS) and third generation sequencing (TGS) technologies.

Hybridisation-based target-enrichment (HBTE) sequencing (also known as capture sequencing) uses target-specific oligonucleotide probes to fragment and tagment the gene region of interest into short reads. These beads are then washed to remove the unbound genomic DNA, before indexes are added via PCR amplification. HBTE offers a number of advantages over ABSR NGS. The use of target-enrichment negates the need for an initial PCR amplification step. As a result, HBTE NGS can provide a result with a minimal amount of DNA (as little as 50ng), and more consistent allele balance in cases of heterozygous alleles, for easier identification of novel alleles in the absence of PCR artifacts. An additional benefit of improved allelic balance is that HBTE can be used to measure CNV, with the number of nucleotide calls generated proportional to the number of copies present. The beads also act as a means of normalisation, making target enrichment robust against inter-sample variation in DNA concentration, reducing the burden of labour in the workflow. Finally, target enrichment sequencing provides a far deeper coverage than is possible via amplicon sequencing, allowing for easier identification of mutations and novel alleles (Norman et al., 2016). Unfortunately, HBTE NGS kits are significantly more expensive than amplicon sequencing. As the technology matures and reduces in cost, it is likely that capture sequencing will supersede short read amplicon sequencing due to the shorter

turnaround time. There is currently no commercially available HBTE NGS kits for sequencing KIR, though several labs have developed their own in-house kits (personal communication).

TGS (also known as long-range PCR-based sequencing, or long-read sequencing) generates amplicons up to 40kb in length, reducing the importance of phasing and facilitating whole gene region reads (Pollard et al., 2018). The PacBio TGS platform uses Single Molecule Real-Time (SMRT) sequencing technology, incorporating hairpin adaptors at the periphery of the amplicon to form a contiguous loop of sequence which can read elongated continuously for sequencing (Schadt et al., 2010; Ardui et al., 2018). SMRT TGS technology is capable is reading lengths spanning entire KIR haplotypes, presenting conclusive designation of KIR genes within their haplotypes, including the definition of CNV (Roe et al., 2017). The detail provided by this technology illuminates KIR in a way previously impossible, providing direct reads for haplotype data that was previously imputed. Unfortunately, the current cost of TGS technology is prohibitively expensive without an extremely high through-put, and the length of the read reduces the sequencing yield, thus reducing read depth and increasing the likelihood of artifacts due to polymerase fidelity (Jia et al., 2014; Ribble et al., 2015). Due to the high economy of scale, TGS is currently only suited to the HPCT donor registries, with NGS more appropriate for most localised clinical laboratories. It is likely that the value of introducing higher resolution testing may exert additional pressure on medical systems to centralise their localised services into regional sequencing ‘supercentres’. Similar to HBTE NGS, there is currently no commercially available TGS kit for sequencing KIR, with some laboratories developing their own in-house kit.

1.5.3 Challenges in NGS of KIR

The primary difficulty in using NGS to sequence the KIR region is in the genes' poor mappability – the accuracy of high resolution sequencing without false base readings (Li and Freudenberg, 2014). In the case of KIR genes, this is largely due to the accuracy of phasing, dependent upon the length of the read (longer reads will be more likely to contain a unique identifiable nucleotide motif) and the degree to which the reads contain repetitive sequences (if identical sequences occur multiple times in a region, analysis software may struggle to phase the reads together accurately) (Li and Freudenberg, 2014; Li et al., 2014). The diversity of many KIR genes and their alleles, arising from meiotic recombination between genes, resulting in a high degree of homology between KIR genes (Martin et al., 2003; Norman et al., 2009). This homology throughout the gene region greatly reduces the overall mappability of KIR.

The presence or absence of KIR genes and CNV also makes it difficult to reliably and accurately impute haplotypes from diplotype data. Short read sequencing can determine whether a KIR allele is present but cannot be used to assign discrete haplotypes, greatly constraining the value of population analysis of KIR genetics by being reliant upon imputation for assignment, which in many cases may be inaccurate. For example, if two copies of a gene are identified in CNV analysis, this may be because a single copy is present on both haplotypes or a single haplotype possesses two copies. These issues are largely overcome by use of TGS, providing a means of sequencing across the whole gene cluster, including the 10kb region of repeating bases between KIR3DP1 and 2DL4, negative the requirement of phasing in analysis (Roe et al., 2017). This technique has identified several novel haplotypes which had been previously incorrectly imputed from other sequencing methodologies. Alternately,

some groups have constructed custom algorithms for imputing most likely haplotype combinations from diplotype data (Vierra-Green et al., 2016).

1.5.4 Reporting of NGS data

The vast volumes of data generated by NGS technology has brought into question the relevancy of current sequencing information formats, such as FASTA, FASTQ and Variant Call Format (VCF). To this end, the Immunogenomics Data Analysis Working Group (IDAWG) formed the Immunogenomic NGS Data Consortium (INGSDC) (Anonymous, 2014) to consolidate knowledge of current sequencing formats into a novel (and future-proof) format that can efficiently support the information required for storing NGS results, as well as to discuss internationally acceptable standards for the Minimum Information for Reporting Immunogenomic NGS Genotyping (MIRING) (Mack et al., 2015). The described aims of the MIRING project are quoted in Figure 11.

As part of the creative process in developing software to support MIRING, a programme of Data Standards Hackathon events (DaSHs) have been coordinated (Brelsford, 2016), organised by the US Food & Drug Administration (FDA) Workshop in collaboration with the National Marrow Donor Program (NMDP). MIRING was also a focus of the 17th International Histocompatibility and Immunogenetics Workshop (IHIW) (held in San Francisco, USA in September 2017), as part of the IHIW's commitment to advancing immunogenomic data management (Misra et al., 2018).

1. *To facilitate downstream analyses and data management for current research and clinical use cases for molecular genotyping data in the histocompatibility and immunogenetics field.*
2. *To permit the re-analysis of NGS HLA or KIR genotyping results in the context of past, present and (foreseeable) future molecular nomenclatures and methods of describing HLA and KIR allele diversity.*
3. *To permit the comparison and evaluation of genotyping performance between different NGS platforms and analysis methods.*
4. *To enable molecular genotyping results generated using SBT, SSOP and SSP genotyping technologies to be incorporated if required.*
5. *That the MIRING elements be sufficient to permit the accurate reporting of NGS data generated for other highly-polymorphic regions of the human genome.*

Figure 11 – Quoted aims of the Minimum Information for Reporting Immunogenomic NGS Genotyping guidelines (Mack et al., 2015)

These developments in NGS reporting systems have brought about a new wave of innovation in the programming behind NGS, such as the Histoimmunogenetics Markup Language (HML) 1.0 format (Milius et al., 2015), which was developed in accordance with the MIRING reporting guidelines. HML 1.0 is both fully backwards compatible and future-proofed, allowing the reporting of SSO, SSP and Sanger genomic sequencing technologies alongside analysis of consensus data provided by NGS.

1.6 Conclusion

The study of KIR has progressed dramatically over the past ten years, transforming our understanding of the role of NK cells in medical science, including the HPCT setting. NGS and other emerging technologies will now help the scientific community to further understand the basic science underlying the function of NK cells, and aid development of new clinical interventions to improve the therapeutic value of a wide range of medical services.

The role of NGS in routine clinical HPCT donor assessments is still unclear. It is possible that the broad gene presence/absence approach of current donor selection

algorithms based on KIR gene content are the optimal balance between clinical efficacy, efficiency of testing, and fiscal pressures. The additional financial burden of NGS coupled with relatively long test turnaround times dictate that high-resolution results must provide a significant improvement in clinical outcomes to favour this technology over low-resolution technologies with a rapid turnaround time, such as PCR-SSO and qPCR. Nonetheless, the use of NGS analysis, cellular and molecular assays in combination offers an exciting opportunity for investigation into the interaction between KIR and HLA and how this relates to the proteomics and cellular function of NK cells. The current research into allelic variation within each KIR gene offers a glimpse into the deeper functions of KIR proteins, but further work is required to elucidate how this variation influences the functional capacity of different NK cell subpopulations.

Finally, despite great developments in the research of KIR, there remains a less than universal usage of a common nomenclature. Care must be taken to ensure published reference sets are used in published studies wherever possible to improve clarity when comparing studies. Further expansion of the work of Pyo et al (2010) and Vierra-Green et al (2012) to designate and apply a universal KIR haplotype nomenclature system would greatly aid this endeavour.

Chapter 1 References

Alicata, C., Pende, D., Meazza, R., Canevali, P., Loiacono, F., Bertaina, A., Locatelli, F., Nemat-Gorgani, N., Guethlein, L. A., Parham, P., Moretta, L., Moretta, A., Bottino, C., Norman, P. J. & Falco, M. (2016) Hematopoietic stem cell transplantation: Improving alloreactive Bw4 donor selection by genotyping codon 86 of KIR3DL1/S1. *Eur J Immunol,* 46(6), 1511-7.

Anonymous. (2014) *Immunogenomic Next Generation Sequencing Data Consortium.* [Online]. IDAWG. Available: http://igdawg.org/ngs.html [Accessed 16/05/2019].

Ardui, S., Ameur, A., Vermeesch, J. R. & Hestand, M. S. (2018) Single molecule real-time (SMRT) sequencing comes of age: applications and utilities for medical diagnostics. *Nucleic Acids Res,* 46(5), 2159-68.

Arima, N., Kanda, J., Tanaka, J., Yabe, T., Morishima, Y., Kim, S. W., Najima, Y., Ozawa, Y., Eto, T., Kanamori, H., Mori, T., Kobayashi, N., Kondo, T., Nakamae, H., Uchida, N., Inoue, M., Fukuda, T., Ichinohe, T., Atsuta, Y. & Kanda, Y. (2018) Homozygous HLA-C1 is associated with reduced risk of relapse after HLA-matched transplantation in patients with myeloid leukemia. *Biol Blood Marrow Transplant,* 24(4), 717-25.

Bao, X., Wang, M., Zhou, H., Zhang, H., Wu, X. & He, J. (2015) Donor KIR genotype Bx1 had a negative effect while centromeric B-specific gene motifs had a positive effect of survival for standard risk AML Patients after unrelated donor hemotopoietic stem cell transplantation. *Human Immunology,* 76.

Bari, R., Rujkijyanont, P., Sullivan, E., Kang, G., Turner, V., Gan, K. & Leung, W. (2013) Effect of donor KIR2DL1 allelic polymorphism on the outcome of pediatric allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol,* 31(30), 3782-90.

Bell, J. C. & Kowalczykowski, S. C. (2016) RecA: Regulation and Mechanism of a Molecular Search Engine. *Trends Biochem Sci,* 41(6), 491-507.

Béziat, V., Traherne, J. A., Liu, L. L., Jayaraman, J., Enqvist, M., Larsson, S., Trowsdale, J. & Malmberg, K. J. (2013) Influence of KIR gene copy number on natural killer cell education. *Blood,* 121(23), 4703-7.

Boelen, L., Debebe, B., Silveira, M., Salam, A., Makinde, J., Roberts, C. H., Wang, E. C. Y., Frater, J., Gilmour, J., Twigger, K., Ladell, K., Miners, K. L., Jayaraman, J., Traherne, J. A., Price, D. A., Qi, Y., Martin, M. P., Macallan, D. C., Thio, C. L., Astemborski, J., Kirk, G., Donfield, S. M., Buchbinder, S., Khakoo, S. I., Goedert, J. J., Trowsdale, J., Carrington, M., Kollnberger, S. & Asquith, B. (2018) Inhibitory killer cell immunoglobulin-like receptors strengthen CD8(+) T cell-mediated control of HIV-1, HCV, and HTLV-1. *Sci Immunol,* 3(29), eaao2892.

Boudreau, J. E., Giglio, F., Gooley, T. A., Stevenson, P. A., Le Luduec, J. B., Shaffer, B. C., Rajalingam, R., Hou, L., Hurley, C. K., Noreen, H., Reed, E. F., Yu, N., Vierra-Green, C., Haagenson, M., Malkki, M., Petersdorf, E. W., Spellman, S. & Hsu, K. C. (2017) KIR3DL1/HLA-B Subtypes Govern Acute Myelogenous Leukemia Relapse After Hematopoietic Cell Transplantation. *J Clin Oncol,* 35(20), 2268-2278.

Boudreau, J. E. & Hsu, K. C. (2018) Natural Killer Cell Education and the Response to Infection and Cancer Therapy: Stay Tuned. *Trends Immunol,* 39(3), 222-239.

Boudreau, J. E., Mulrooney, T. J., Le Luduec, J. B., Barker, E. & Hsu, K. C. (2016) KIR3DL1 and HLA-B density and binding calibrate NK education and response to HIV. *J Immunol,* 196(8), 3398-410.

Boyington, J. C., Motyka, S. A., Schuck, P., Brooks, A. G. & Sun, P. D. (2000) Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. *Nature,* 405(6786), 537-43.

Brelsford, J. (2016) *NMDP-bioinformatics/DaSH Home.* [Online]. Available: https://github.com/nmdp-bioinformatics/dash/wiki [Accessed 16/05/2019].

Burek Kamenaric, M., Stingl Jankovic, K., Grubic, Z., Serventi Seiwerth, R., Maskalan, M., Nemet, D., Mikulic, M. & Zunec, R. (2017) The impact of KIR2DS4 gene on clinical outcome after hematopoietic stem cell transplantation. *Hum Immunol,* 78(2), 95-102.

Burian, A., Wang, K. L., Finton, K. A., Lee, N., Ishitani, A., Strong, R. K. & Geraghty, D. E. (2016) HLA-F and MHC-I open conformers bind natural killer cell Ig-like receptor KIR3DS1. *PLoS One,* 11(9), e0163297.

Canter, R. J., Grossenbacher, S. K., Foltz, J. A., Sturgill, I. R., Park, J. S., Luna, J. I., Kent, M. S., Culp, W. T. N., Chen, M., Modiano, J. F., Monjazeb, A. M., Lee, D. A. & Murphy, W. J. (2017) Radiotherapy enhances natural killer cell cytotoxicity and localization in pre-clinical canine sarcomas and first-in-dog clinical trial. *J Immunother Cancer,* 5(1), 98.

Carapito, R., Radosavljevic, M. & Bahram, S. (2016) Next-generation sequencing of the HLA locus: methods and impacts on HLA typing, population genetics and disease association studies. *Hum Immunol,* 77(11), 1016-23.

Cella, M., Longo, A., Ferrara, G. B., Strominger, J. L. & Colonna, M. (1994) NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. *J Exp Med,* 180(4), 1235-42.

Chan, H. W., Kurago, Z. B., Stewart, C. A., Wilson, M. J., Martin, M. P., Mace, B. E., Carrington, M., Trowsdale, J. & Lutz, C. T. (2003) DNA methylation maintains allele-specific KIR gene expression in human natural killer cells. *J Exp Med,* 197(2), 245-55.

Chan, Y. L. T., Zuo, J., Inman, C., Croft, W., Begum, J., Croudace, J., Kinsella, F., Maggs, L., Nagra, S., Nunnick, J., Abbotts, B., Craddock, C., Malladi, R. & Moss, P. (2018) NK cells produce high levels of IL-10 early after allogeneic stem cell transplantation and suppress development of acute GVHD. *Eur J Immunol,* 48(2), 316-329.

Chen, Y., Shi, Y., Cheng, H., An, Y. Q. & Gao, G. F. (2009) Structural immunology and crystallography help immunologists see the immune system in action: how T and NK cells touch their ligands. *IUBMB Life,* 61(6), 579-90.

Ciccone, E., Pende, D., Nanni, L., Di Donato, C., Viale, O., Beretta, A., Vitale, M., Sivori, S., Moretta, A. & Moretta, L. (1995) General role of HLA class I molecules in the protection of target cells from lysis by natural killer cells: evidence that the free heavy chains of class I molecules are not sufficient to mediate the protective effect. *Int Immunol,* 7(3), 393-400.

Cichocki, F., Miller, J. S. & Anderson, S. K. (2011) Killer immunoglobulin-like receptor transcriptional regulation: a fascinating dance of multiple promoters. *J Innate Immun,* 3(3), 242-8.

Colonna, M., Borsellino, G., Falco, M., Ferrara, G. B. & Strominger, J. L. (1993) HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci U S A,* 90(24), 12000-4.

Colonna, M., Spies, T., Strominger, J. L., Ciccone, E., Moretta, A., Moretta, L., Pende, D. & Viale, O. (1992) Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene. *Proc Natl Acad Sci U S A,* 89(17), 7983-5.

Cooley, S., Trachtenberg, E., Bergemann, T. L., Saeteurn, K., Klein, J., Le, C. T., Marsh, S. G., Guethlein, L. A., Parham, P., Miller, J. S. & Weisdorf, D. J. (2009) Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood,* 113(3), 726-32.

Cooley, S., Weisdorf, D. J., Guethlein, L. A., Klein, J. P., Wang, T., Le, C. T., Marsh, S. G., Geraghty, D., Spellman, S., Haagenson, M. D., Ladner, M., Trachtenberg, E., Parham, P. & Miller, J. S. (2010) Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood,* 116(14), 2411-9.

Cooley, S., Weisdorf, D. J., Guethlein, L. A., Klein, J. P., Wang, T., Marsh, S. G., Spellman, S., Haagenson, M. D., Saeturn, K., Ladner, M., Trachtenberg, E., Parham, P. & Miller, J. S. (2014) Donor killer cell Ig-like receptor B haplotypes, recipient HLA-C1, and HLA-C mismatch enhance the clinical benefit of unrelated transplantation for acute myelogenous leukemia. *J Immunol,* 192(10), 4592-600.

Davies, G. E., Locke, S. M., Wright, P. W., Li, H., Hanson, R. J., Miller, J. S. & Anderson, S. K. (2007) Identification of bidirectional promoters in the human KIR genes. *Genes Immun,* 8(3), 245-53.

Dulberger, C. L., McMurtrey, C. P., Holzemer, A., Neu, K. E., Liu, V., Steinbach, A. M., Garcia-Beltran, W. F., Sulak, M., Jabri, B., Lynch, V. J., Altfeld, M., Hildebrand, W. H. & Adams, E. J. (2017) Human leukocyte antigen F presents peptides and regulates immunity through interactions with NK cell receptors. *Immunity,* 46(6), 1018-29 e7.

EMBL-EBI. (2017) *Donor KIR B-content group calculator* [Online]. Available: https://www.ebi.ac.uk/ipd/kir/donor_b_content.html [Accessed 16/05/2019].

EMBL-EBI. (2019) *Statistics for release 2.9.0 (December 2019)* [Online]. Available: https://www.ebi.ac.uk/ipd/kir/stats.html [Accessed 16/05/2019].

Fan, Q. R., Garboczi, D. N., Winter, C. C., Wagtmann, N., Long, E. O. & Wiley, D. C. (1996) Direct binding of a soluble natural killer cell inhibitory receptor to a soluble human leukocyte antigen-Cw4 class I major histocompatibility complex molecule. *Proc Natl Acad Sci U S A,* 93(14), 7178-83.

Fan, Q. R., Long, E. O. & Wiley, D. C. (2001) Crystal structure of the human natural killer cell inhibitory receptor KIR2DL1-HLA-Cw4 complex. *Nat Immunol,* 2(5), 452-60.

Fan, Q. R., Mosyak, L., Winter, C. C., Wagtmann, N., Long, E. O. & Wiley, D. C. (1997) Structure of the inhibitory receptor for human natural killer cells resembles haematopoietic receptors. *Nature,* 389(6646), 96-100.

Fan, Q. R. & Wiley, D. C. (1999) Structure of human histocompatibility leukocyte antigen (HLA)-Cw4, a ligand for the KIR2D natural killer cell inhibitory receptor. *J Exp Med,* 190(1), 113-23.

Faridi, R. M., Kemp, T. J., Dharmani-Khan, P., Lewis, V., Tripathi, G., Rajalingam, R., Daly, A., Berka, N., Storek, J. & Masood Khan, F. (2016) Donor-Recipient Matching for KIR Genotypes Reduces Chronic GVHD and Missing Inhibitory KIR Ligands Protect against Relapse after Myeloablative, HLA Matched Hematopoietic Cell Transplantation. *PLoS One,* 11(6), e0158242.

Fischer, J. C., Ottinger, H., Ferencik, S., Sribar, M., Punzel, M., Beelen, D. W., Schwan, M. A., Grosse-Wilde, H., Wernet, P. & Uhrberg, M. (2007) Relevance of C1 and C2 epitopes for hemopoietic stem cell transplantation: role for sequential acquisition of HLA-C-specific inhibitory killer Ig-like receptor. *The Journal of Immunology,* 178(6), 3918-23.

Frazier, W. R., Steiner, N., Hou, L., Dakshanamurthy, S. & Hurley, C. K. (2013) Allelic variation in KIR2DL3 generates a KIR2DL2-like receptor with increased binding to its HLA-C ligand. *J Immunol,* 190(12), 6198-208.

Gamazon, E. R. & Stranger, B. E. (2015) The impact of human copy number variation on gene expression. *Brief Funct Genomics,* 14(5), 352-7.

Gendzekhadze, K., Garcia-Gomez, J., Cano, P. & Senitzer, D. (2015) KIR shapes HLA. *Human Immunology,* 76, 40.

Gianchecchi, E., Delfino, D. V. & Fierabracci, A. (2018) NK cells in autoimmune diseases: Linking innate and adaptive immune responses. *Autoimmun Rev,* 17(2), 142-54.

Gomez-Lozano, N., Estefania, E., Williams, F., Halfpenny, I., Middleton, D., Solis, R. & Vilches, C. (2005) The silent KIR3DP1 gene (CD158c) is transcribed and might encode a secreted receptor in a minority of humans, in whom the KIR3DP1, KIR2DL4 and KIR3DL1/KIR3DS1 genes are duplicated. *Eur J Immunol,* 35(1), 16-24.

Gonzalez-Galarza, F. F., Takeshita, L. Y., Santos, E. J., Kempson, F., Maia, M. H., da Silva, A. L., Teles e Silva, A. L., Ghattaoraya, G. S., Alfirevic, A., Jones, A. R. & Middleton, D. (2015) Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. *Nucleic Acids Res,* 43(Database issue), D784-8.

Hansasuta, P., Dong, T., Thananchai, H., Weekes, M., Willberg, C., Aldemir, H., Rowland-Jones, S. & Braud, V. M. (2004) Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. *Eur J Immunol,* 34(6), 1673-9.

Hilton, H. G., Guethlein, L. A., Goyos, A., Nemat-Gorgani, N., Bushnell, D. A., Norman, P. J. & Parham, P. (2015a) Polymorphic HLA-C receptors balance the functional characteristics of KIR haplotypes. *J Immunol,* 195(7), 3160-70.

Hilton, H. G., Norman, P. J., Nemat-Gorgani, N., Goyos, A., Hollenbach, J. A., Henn, B. M., Gignoux, C. R., Guethlein, L. A. & Parham, P. (2015b) Loss and gain of natural killer cell receptor function in an African hunter-gatherer population. *PLoS Genet,* 11(8), e1005439.

Hölzemer, A., Thobakgale, C. F., Jimenez Cruz, C. A., Garcia-Beltran, W. F., Carlson, J. M., van Teijlingen, N. H., Mann, J. K., Jaggernath, M., Kang, S. G., Korner, C., Chung, A. W., Schafer, J. L., Evans, D. T., Alter, G., Walker, B. D., Goulder, P. J., Carrington, M., Hartmann, P., Pertel, T., Zhou, R., Ndung'u, T. & Altfeld, M. (2015) Selection of an HLA-C*03:04-Restricted HIV-1 p24 Gag Sequence Variant Is Associated with Viral Escape from KIR2DL3+ Natural Killer Cells: Data from an Observational Cohort in South Africa. *PLoS Med,* 12(11), e1001900; discussion e1001900.

Horowitz, A., Strauss-Albee, D. M., Leipold, M., Kubo, J., Nemat-Gorgani, N., Dogan, O. C., Dekker, C. L., Mackey, S., Maecker, H., Swan, G. E., Davis, M. M., Norman, P. J., Guethlein, L. A., Desai, M., Parham, P. & Blish, C. A. (2013) Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. *Sci Transl Med,* 5(208), 208ra145.

Hou, L., Chen, M., Steiner, N., Kariyawasam, K., Ng, J. & Hurley, C. K. (2012) Killer cell immunoglobulin-like receptors (KIR) typing by DNA sequencing. *Methods Mol Biol,* 882**,** 431-68.

Howard, C. A., Fernandez-Vina, M. A., Appelbaum, F. R., Confer, D. L., Devine, S. M., Horowitz, M. M., Mendizabal, A., Laport, G. G., Pasquini, M. C. & Spellman, S. R. (2015) Recommendations for donor human leukocyte antigen assessment and matching for allogeneic stem cell transplantation: consensus opinion of the Blood and Marrow Transplant Clinical Trials Network (BMT CTN). *Biol Blood Marrow Transplant,* 21(1)**,** 4-7.

Hsu, K. C., Chida, S., Geraghty, D. E. & Dupont, B. (2002a) The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev,* 190**,** 40-52.

Hsu, K. C., Liu, X. R., Selvakumar, A., Mickelson, E., O'Reilly, R. J. & Dupont, B. (2002b) Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *The Journal of Immunology,* 169(9)**,** 5118-29.

Jia, H., Guo, Y., Zhao, W. & Wang, K. (2014) Long-range PCR in next-generation sequencing: comparison of six enzymes and evaluation on the MiSeq sequencer. *Sci Rep,* 4(5737)**,** 1-8.

Jiang, W., Johnson, C., Jayaraman, J., Simecek, N., Noble, J., Moffatt, M. F., Cookson, W. O., Trowsdale, J. & Traherne, J. A. (2012) Copy number variation leads to considerable diversity for B but not A haplotypes of the human KIR genes encoding NK cell receptors. *Genome Res,* 22(10)**,** 1845-54.

Kärre, K., Ljunggren, H. G., Piontek, G. & Kiessling, R. (1986) Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature,* 319(6055)**,** 675-8.

Leung, W., Handgretinger, R., Iyengar, R., Turner, V., Holladay, M. S. & Hale, G. A. (2007) Inhibitory KIR-HLA receptor-ligand mismatch in autologous haematopoietic stem cell transplantation for solid tumour and lymphoma. *Br J Cancer,* 97(4)**,** 539-42.

Leung, W., Iyengar, R., Turner, V., Lang, P., Bader, P., Conn, P., Niethammer, D. & Handgretinger, R. (2004) Determinants of antileukemia effects of allogeneic NK cells. *The Journal of Immunology,* 172(1)**,** 644-50.

Li, W. & Freudenberg, J. (2014) Mappability and read length. *Front Genet,* 5(381)**,** 1-7.

Li, W., Freudenberg, J. & Miramontes, P. (2014) Diminishing return for increased mappability with longer sequencing reads: implications of the k-mer distributions in the human genome. *BMC Bioinformatics,* 15(2)**,** 1-12.

Li, Y. & Mariuzza, R. A. (2014) Structural basis for recognition of cellular and viral ligands by NK cell receptors. *Front Immunol,* 5**,** 123.

Liu, J., Xiao, Z., Ko, H. L., Shen, M. & Ren, E. C. (2014) Activating killer cell immunoglobulin-like receptor 2DS2 binds to HLA-A*11. *Proc Natl Acad Sci U S A,* 111(7)**,** 2662-7.

Lunemann, S., Martrus, G., Holzemer, A., Chapel, A., Ziegler, M., Korner, C., Garcia Beltran, W., Carrington, M., Wedemeyer, H. & Altfeld, M. (2016) Sequence variations in HCV core-derived epitopes alter binding of KIR2DL3 to HLA-C *03:04 and modulate NK cell function. *J Hepatol,* 65(2)**,** 252-8.

Mack, S. J., Milius, R. P., Gifford, B. D., Sauter, J., Hofmann, J., Osoegawa, K., Robinson, J., Groeneweg, M., Turenchalk, G. S., Adai, A., Holcomb, C., Rozemuller, E. H., Penning, M. T., Heuer, M. L., Wang, C., Salit, M. L.,

Schmidt, A. H., Parham, P. R., Muller, C., Hague, T., Fischer, G., Fernandez-Vina, M., Hollenbach, J. A., Norman, P. J. & Maiers, M. (2015) Minimum information for reporting next generation sequence genotyping (MIRING): guidelines for reporting HLA and KIR genotyping via next generation sequencing. *Hum Immunol,* 76(12), 954-62.

Mandelboim, O., Reyburn, H. T., Valés-Gómez, M., Pazmany, L., Colonna, M., Borsellino, G. & Strominger, J. L. (1996) Protection from lysis by Natural Killer cells of group 1 and 2 specificity is mediated by residue 80 in human Histocompatibility Leukocyte Antigen C alleles and also occurs with empty Major Histocompatibility Complex molecules. *J Exp Med,* 184, 913-22.

Manser, A. R., Weinhold, S. & Uhrberg, M. (2015) Human KIR repertoires: shaped by genetic diversity and evolution. *Immunol Rev,* 267(1), 178-96.

Marsh, S. G., Parham, P., Dupont, B., Geraghty, D. E., Trowsdale, J., Middleton, D., Vilches, C., Carrington, M., Witt, C., Guethlein, L. A., Shilling, H., Garcia, C. A., Hsu, K. C. & Wain, H. (2003) Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Immunogenetics,* 55(4), 220-6.

Martin, M. P., Bashirova, A., Traherne, J., Trowsdale, J. & Carrington, M. (2003) Cutting edge: expansion of the KIR locus by unequal crossing over. *The Journal of Immunology,* 171(5), 2192-5.

Martin, M. P., Gao, X., Lee, J. H., Nelson, G. W., Detels, R., Goedert, J. J., Buchbinder, S., Hoots, K., Vlahov, D., Trowsdale, J., Wilson, M., O'Brien, S. J. & Carrington, M. (2002) Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet,* 31(4), 429-34.

Martin, M. P., Naranbhai, V., Shea, P. R., Qi, Y., Ramsuran, V., Vince, N., Gao, X., Thomas, R., Brumme, Z. L., Carlson, J. M., Wolinsky, S. M., Goedert, J. J., Walker, B. D., Segal, F. P., Deeks, S. G., Haas, D. W., Migueles, S. A., Connors, M., Michael, N., Fellay, J., Gostick, E., Llewellyn-Lacey, S., Price, D. A., Lafont, B. A., Pymm, P., Saunders, P. M., Widjaja, J., Wong, S. C., Vivian, J. P., Rossjohn, J., Brooks, A. G. & Carrington, M. (2018) Killer cell immunoglobulin-like receptor 3DL1 variation modifies HLA-B*57 protection against HIV-1. *J Clin Invest,* 128(5), 1903-1912.

Martin, M. P., Qi, Y., Gao, X., Yamada, E., Martin, J. N., Pereyra, F., Colombo, S., Brown, E. E., Shupert, W. L., Phair, J., Goedert, J. J., Buchbinder, S., Kirk, G. D., Telenti, A., Connors, M., O'Brien, S. J., Walker, B. D., Parham, P., Deeks, S. G., McVicar, D. W. & Carrington, M. (2007) Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet,* 39(6), 733-40.

Maxwell, L. D., Wallace, A., Middleton, D. & Curran, M. D. (2002) A common KIR2DS4 deletion variant in the human that predicts a soluble KIR molecule analogous to the KIR1D molecule observed in the rhesus monkey. *Tissue Antigens,* 60(3), 254-8.

Mayor, N. P., Hayhurst, J. D., Turner, T. R., Szydlo, R. M., Shaw, B. E., Bultitude, W. P., Sayno, J. R., Tavarozzi, F., Latham, K., Anthias, C., Robinson, J., Braund, H., Danby, R., Perry, J., Wilson, M. C., Bloor, A. J., McQuaker, I. G., MacKinnon, S., Marks, D. I., Pagliuca, A., Potter, M. N., Potter, V. T., Russell, N. H., Thomson, K. J., Madrigal, J. A. & Marsh, S. G. E. (2019) Recipients Receiving Better HLA-Matched Hematopoietic Cell Transplantation Grafts, Uncovered by a Novel HLA Typing Method, Have Superior Survival: A Retrospective Study. *Biol Blood Marrow Transplant,* 25(3), 443-450.

Middleton, D., Gonzalez, A. & Gilmore, P. M. (2007) Studies on the expression of the deleted KIR2DS4*003 gene product and distribution of KIR2DS4 deleted and nondeleted versions in different populations. *Hum Immunol,* 68(2), 128-34.

Milius, R. P., Heuer, M., Valiga, D., Doroschak, K. J., Kennedy, C. J., Bolon, Y. T., Schneider, J., Pollack, J., Kim, H. R., Cereb, N., Hollenbach, J. A., Mack, S. J. & Maiers, M. (2015) Histoimmunogenetics Markup Language 1.0: reporting next generation sequencing-based HLA and KIR genotyping. *Hum Immunol,* 76(12), 963-74.

Miller, J. S. & McCullar, V. (2001) Human natural killer cells with polyclonal lectin and immunoglobulinlike receptors develop from single hematopoietic stem cells with preferential expression of NKG2A and KIR2DL2/L3/S2. *Blood,* 98(3), 705-13.

Minculescu, L., Marquart, H. V., Friis, L. S., Petersen, S. L., Schiodt, I., Ryder, L. P., Andersen, N. S. & Sengeloev, H. (2016) Early Natural Killer Cell Reconstitution Predicts Overall Survival in T Cell-Replete Allogeneic Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant,* 22(12), 2187-2193.

Misra, M. K., Augusto, D. G., Martin, G. M., Nemat-Gorgani, N., Sauter, J., Hofmann, J. A., Traherne, J. A., Gonzalez-Quezada, B., Gorodezky, C., Bultitude, W. P., Marin, W., Vierra-Green, C., Anderson, K. M., Balas, A., Caro-Oleas, J. L., Cisneros, E., Colucci, F., Dandekar, R., Elfishawi, S. M., Fernandez-Vina, M. A., Fouda, M., Gonzalez-Fernandez, R., Grosse, A., Herrero-Mata, M. J., Hollenbach, S. Q., Marsh, S. G. E., Mentzer, A., Middleton, D., Moffett, A., Moreno-Hidalgo, M. A., Mossallam, G. I., Nakimuli, A., Oksenberg, J. R., Oppenheimer, S. J., Parham, P., Petzl-Erler, M. L., Planelles, D., Sanchez-Garcia, F., Sanchez-Gordo, F., Schmidt, A. H., Trowsdale, J., Vargas, L. B., Vicario, J. L., Vilches, C., Norman, P. J. & Hollenbach, J. A. (2018) Report from the killer-cell immunoglobulin-like receptors (KIR) component of the 17th International HLA and Immunogenetics Workshop. *Hum Immunol,* 79(12), 825-33.

Moesta, A. K., Norman, P. J., Yawata, M., Yawata, N., Gleimer, M. & Parham, P. (2008) Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *The Journal of Immunology,* 180(6), 3969-79.

Nelson, W. C., Pyo, C. W., Vogan, D., Wang, R., Pyon, Y. S., Hennessey, C., Smith, A., Pereira, S., Ishitani, A. & Geraghty, D. E. (2015) An integrated genotyping approach for HLA and other complex genetic systems. *Hum Immunol,* 76(12), 928-38.

Neuchel, C., Furst, D., Niederwieser, D., Bunjes, D., Tsamadou, C., Wulf, G., Pfreundschuh, M., Wagner, E., Stuhler, G., Einsele, H., Schrezenmeier, H. & Mytilineos, J. (2017) Impact of donor activating KIR genes on HSCT outcome in C1-ligand negative myeloid disease patients transplanted with unrelated donors - a retrospective study. *PLoS One,* 12(1), e0169512.

Nguyen, S., Dhedin, N., Vernant, J. P., Kuentz, M., Al Jijakli, A., Rouas-Freiss, N., Carosella, E. D., Boudifa, A., Debre, P. & Vieillard, V. (2005) NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. *Blood,* 105(10), 4135-42.

Norman, P. J., Abi-Rached, L., Gendzekhadze, K., Hammond, J. A., Moesta, A. K., Sharma, D., Graef, T., McQueen, K. L., Guethlein, L. A., Carrington, C. V.,

Chandanayingyong, D., Chang, Y. H., Crespi, C., Saruhan-Direskeneli, G., Hameed, K., Kamkamidze, G., Koram, K. A., Layrisse, Z., Matamoros, N., Mila, J., Park, M. H., Pitchappan, R. M., Ramdath, D. D., Shiau, M. Y., Stephens, H. A., Struik, S., Tyan, D., Verity, D. H., Vaughan, R. W., Davis, R. W., Fraser, P. A., Riley, E. M., Ronaghi, M. & Parham, P. (2009) Meiotic recombination generates rich diversity in NK cell receptor genes, alleles, and haplotypes. *Genome Res,* 19(5), 757-69.

Norman, P. J., Hollenbach, J. A., Nemat-Gorgani, N., Marin, W. M., Norberg, S. J., Ashouri, E., Jayaraman, J., Wroblewski, E. E., Trowsdale, J., Rajalingam, R., Oksenberg, J. R., Chiaroni, J., Guethlein, L. A., Traherne, J. A., Ronaghi, M. & Parham, P. (2016) Defining KIR and HLA class I genotypes at highest resolution via high-throughput sequencing. *Am J Hum Genet,* 99(2), 375-91.

Nowak, J., Koscinska, K., Mika-Witkowska, R., Rogatko-Koros, M., Mizia, S., Jaskula, E., Polak, M., Mordak-Domagala, M., Lange, J., Gronkowska, A., Jedrzejczak, W. W., Kyrcz-Krzemien, S., Markiewicz, M., Dzierzak-Mietla, M., Tomaszewska, A., Nasilowska-Adamska, B., Szczepinski, A., Halaburda, K., Hellmann, A., Czyz, A., Gil, L., Komarnicki, M., Wachowiak, J., Baranska, M., Kowalczyk, J., Drabko, K., Gozdzik, J., Wysoczanska, B., Bogunia-Kubik, K., Graczyk-Pol, E., Witkowska, A., Marosz-Rudnicka, A., Nestorowicz, K., Dziopa, J., Szlendak, U., Warzocha, K., Lange, A. & Polish Donor-Recipient Matching Study, G. (2015) Role of donor activating KIR-HLA ligand-mediated NK cell education status in control of malignancy in hematopoietic cell transplant recipients. *Biol Blood Marrow Transplant,* 21(5), 829-39.

Nowak, J., Koscinska, K., Mika-Witkowska, R., Rogatko-Koros, M., Mizia, S., Jaskula, E., Polak, M., Mordak-Domagala, M., Lange, J., Gronkowska, A., Jedrzejczak, W. W., Kyrcz-Krzemien, S., Markiewicz, M., Dzierzak-Mietla, M., Tomaszewska, A., Nasilowska-Adamska, B., Szczepinski, A., Halaburda, K., Hellmann, A., Komarnicki, M., Gil, L., Czyz, A., Wachowiak, J., Baranska, M., Kowalczyk, J., Drabko, K., Gozdzik, J., Wysoczanska, B., Bogunia-Kubik, K., Graczyk-Pol, E., Witkowska, A., Marosz-Rudnicka, A., Nestorowicz, K., Dziopa, J., Szlendak, U., Warzocha, K., Lange, A. A. & Polish Donor-Recipient Matching Study, G. (2014) Donor NK cell licensing in control of malignancy in hematopoietic stem cell transplant recipients. *Am J Hematol,* 89(10), E176-83.

O'Connor, G. M., Vivian, J. P., Gostick, E., Pymm, P., Lafont, B. A., Price, D. A., Rossjohn, J., Brooks, A. G. & McVicar, D. W. (2015) Peptide-dependent recognition of HLA-B*57:01 by KIR3DS1. *J Virol,* 89(10), 5213-21.

Oevermann, L., Michaelis, S. U., Mezger, M., Lang, P., Toporski, J., Bertaina, A., Zecca, M., Moretta, L., Locatelli, F. & Handgretinger, R. (2014) KIR B haplotype donors confer a reduced risk for relapse after haploidentical transplantation in children with ALL. *Blood,* 124(17), 2744-7.

Pando, M. J., Gardiner, C. M., Gleimer, M., McQueen, K. L. & Parham, P. (2003) The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. *The Journal of Immunology,* 171(12), 6640-9.

Parham, P., Norman, P. J., Abi-Rached, L. & Guethlein, L. A. (2012) Human-specific evolution of killer cell immunoglobulin-like receptor recognition of major histocompatibility complex class I molecules. *Philos Trans R Soc Lond B Biol Sci,* 367(1590), 800-11.

Park, H., Rho, E. Y., In, J. W., Kim, I., Yoon, S. S., Park, S., Shin, S., Park, K. U. & Song, E. Y. (2015a) The impact of HLA and KIR ligand mismatching on unrelated allogeneic hematopoietic stem cell transplantation in Korean adult patients. *Ann Lab Med,* 35(1), 111-7.

Park, S., Kim, K., Jang, J. H., Kim, S. J., Kim, W. S., Kang, E. S. & Jung, C. W. (2015b) KIR alloreactivity based on the receptor-ligand model is associated with improved clinical outcomes of allogeneic hematopoietic stem cell transplantation: result of single center prospective study. *Hum Immunol,* 76(9), 636-43.

Pende, D., Falco, M., Vitale, M., Cantoni, C., Vitale, C., Munari, E., Bertaina, A., Moretta, F., Del Zotto, G., Pietra, G., Mingari, M. C., Locatelli, F. & Moretta, L. (2019) Killer Ig-Like Receptors (KIRs): Their Role in NK Cell Modulation and Developments Leading to Their Clinical Exploitation. *Front Immunol,* 10, 1179.

Pical-Izard, C., Crocchiolo, R., Granjeaud, S., Kochbati, E., Just-Landi, S., Chabannon, C., Frassati, C., Picard, C., Blaise, D., Olive, D. & Fauriat, C. (2015) Reconstitution of natural killer cells in HLA-matched HSCT after reduced-intensity conditioning: impact on clinical outcome. *Biol Blood Marrow Transplant,* 21(3), 429-39.

Pollard, M. O., Gurdasani, D., Mentzer, A. J., Porter, T. & Sandhu, M. S. (2018) Long reads: their purpose and place. *Hum Mol Genet,* 27(R2), R234-41.

Pyo, C. W., Guethlein, L. A., Vu, Q., Wang, R., Abi-Rached, L., Norman, P. J., Marsh, S. G., Miller, J. S., Parham, P. & Geraghty, D. E. (2010) Different patterns of evolution in the centromeric and telomeric regions of group A and B haplotypes of the human killer cell Ig-like receptor locus. *PLoS One,* 5(12), e15115.

Pyo, C. W., Wang, R., Vu, Q., Cereb, N., Yang, S. Y., Duh, F. M., Wolinsky, S., Martin, M. P., Carrington, M. & Geraghty, D. E. (2013) Recombinant structures expand and contract inter and intragenic diversification at the KIR locus. *BMC Genomics,* 14, 89.

Rajagopalan, S. & Long, E. O. (1997) The direct binding of a p58 killer cell inhibitory receptor to human histocompatibility leukocyte antigen (HLA)-Cw4 exhibits peptide selectivity. *J Exp Med,* 185(8), 1523-8.

Ribble, W., Kane, S. D. & Bullard, J. M. (2015) Long-range PCR amplification of DNA by DNA polymerase III holoenzyme from Thermus thermophilus. *Enzyme Res,* 2015, 837842.

Robinson, J., Barker, D. J., Georgiou, X., Cooper, M. A., Flicek, P. & Marsh, S. G. E. (2020) IPD-IMGT/HLA Database. *Nucleic Acids Res,* 48(D1), D948-D955.

Robinson, J., Halliwell, J. A., Hayhurst, J. D., Flicek, P., Parham, P. & Marsh, S. G. (2015) The IPD and IMGT/HLA database: allele variant databases. *Nucleic Acids Res,* 43(Database issue), D423-31.

Roe, D., Vierra-Green, C., Pyo, C. W., Eng, K., Hall, R., Kuang, R., Spellman, S., Ranade, S., Geraghty, D. E. & Maiers, M. (2017) Revealing complete complex KIR haplotypes phased by long-read sequencing technology. *Genes Immun,* 18(3), 127-34.

Ruggeri, L., Capanni, M., Casucci, M., Volpi, I., Tosti, A., Perruccio, K., Urbani, E., Negrin, R. S., Martelli, M. F. & Velardi, A. (1999) Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood,* 94(1), 333-9.

Sahin, U., Dalva, K., Gungor, F., Ustun, C. & Beksac, M. (2018) Donor-recipient killer immunoglobulin like receptor (KIR) genotype matching has a protective effect on chronic graft versus host disease and relapse incidence following HLA-identical sibling hematopoietic stem cell transplantation. *Ann Hematol,* 97(6), 1027-39.

Sambrook, J. G., Bashirova, A., Andersen, H., Piatak, M., Vernikos, G. S., Coggill, P., Lifson, J. D., Carrington, M. & Beck, S. (2006) Identification of the ancestral killer immunoglobulin-like receptor gene in primates. *BMC Genomics,* 7, 209.

Santourlidis, S., Trompeter, H. I., Weinhold, S., Eisermann, B., Meyer, K. L., Wernet, P. & Uhrberg, M. (2002) Crucial role of DNA methylation in determination of clonally distributed killer cell Ig-like receptor expression patterns in NK cells. *The Journal of Immunology,* 169(8), 4253-61.

Saulquin, X., Gastinel, L. N. & Vivier, E. (2003) Crystal structure of the human natural killer cell activating receptor KIR2DS2 (CD158j). *J Exp Med,* 197(7), 933-8.

Saunders, P. M., MacLachlan, B. J., Pymm, P., Illing, P. T., Deng, Y., Wong, S. C., Oates, C. V. L., Purcell, A. W., Rossjohn, J., Vivian, J. P. & Brooks, A. G. (2020) The molecular basis of how buried human leukocyte antigen polymorphism modulates natural killer cell function. *Proc Natl Acad Sci U S A,* 117(21), 11636-11647.

Saunders, P. M., Vivian, J. P., Baschuk, N., Beddoe, T., Widjaja, J., O'Connor, G. M., Hitchen, C., Pymm, P., Andrews, D. M., Gras, S., McVicar, D. W., Rossjohn, J. & Brooks, A. G. (2015) The interaction of KIR3DL1*001 with HLA class I molecules is dependent upon molecular microarchitecture within the Bw4 epitope. *J Immunol,* 194(2), 781-9.

Schadt, E. E., Turner, S. & Kasarskis, A. (2010) A window into third-generation sequencing. *Hum Mol Genet,* 19(R2), R227-40.

Schafer, J. L., Ries, M., Guha, N., Connole, M., Colantonio, A. D., Wiertz, E. J., Wilson, N. A., Kaur, A. & Evans, D. T. (2015) Suppression of a Natural Killer Cell Response by Simian Immunodeficiency Virus Peptides. *PLoS Pathog,* 11(9), e1005145.

Selvakumar, A., Steffens, U. & Dupont, B. (1996) NK cell receptor gene of the KIR family with two IG domains but highest homology to KIR receptors with three IG domains. *Tissue Antigens,* 48(4 Pt 1), 285-94.

Shaw, B. E., Mayor, N. P., Szydlo, R. M., Bultitude, W. P., Anthias, C., Kirkland, K., Perry, J., Clark, A., Mackinnon, S., Marks, D. I., Pagliuca, A., Potter, M. N., Russell, N. H., Thomson, K., Madrigal, J. A. & Marsh, S. G. E. (2017) Recipient/donor HLA and CMV matching in recipients of T-cell-depleted unrelated donor haematopoietic cell transplants. *Bone Marrow Transplant,* 52(5), 717-725.

Sim, M. J., Malaker, S. A., Khan, A., Stowell, J. M., Shabanowitz, J., Peterson, M. E., Rajagopalan, S., Hunt, D. F., Altmann, D. M., Long, E. O. & Boyton, R. J. (2017) Canonical and Cross-reactive Binding of NK Cell Inhibitory Receptors to HLA-C Allotypes Is Dictated by Peptides Bound to HLA-C. *Front Immunol,* 8, 193.

Sim, M. J. W., Rajagopalan, S., Altmann, D. M., Boyton, R. J., Sun, P. D. & Long, E. O. (2019) Human NK cell receptor KIR2DS4 detects a conserved bacterial epitope presented by HLA-C. *Proc Natl Acad Sci U S A,* 116(26), 12964-12973.

Sims, D., Sudbery, I., Ilott, N. E., Heger, A. & Ponting, C. P. (2014) Sequencing depth and coverage: key considerations in genomic analyses. *Nat Rev Genet,* 15(2), 121-32.

Snyder, G. A., Brooks, A. G. & Sun, P. D. (1999) Crystal structure of the HLA-Cw3 allotype-specific killer cell inhibitory receptor KIR2DL2. *Proc Natl Acad Sci U S A,* 96(7), 3864-9.

Sobecks, R. M., Wang, T., Askar, M., Gallagher, M. M., Haagenson, M., Spellman, S., Fernandez-Vina, M., Malmberg, K. J., Muller, C., Battiwalla, M., Gajewski, J., Verneris, M. R., Ringden, O., Marino, S., Davies, S., Dehn, J., Bornhauser, M., Inamoto, Y., Woolfrey, A., Shaw, P., Pollack, M., Weisdorf, D., Millller, J., Hurley, C., Lee, S. J. & Hsu, K. (2015) Impact of KIR and HLA genotypes on outcomes after reduced-intensity conditioning hematopoietic cell transplantation. *Biol Blood Marrow Transplant,* 21(9), 1589-96.

Stewart, C. A., Laugier-Anfossi, F., Vély, F., Saulquin, X., Riedmuller, J., Tisserant, A., Gauthier, L., Romagné, F., Ferracci, G., Arosa, F. A., Moretta, A., Sun, P. D., Ugolini, S. & Vivier, E. (2005) Recognition of peptide-MHC class I complexes by killer immunoglobulin-like receptors. *Proc Natl Acad Sci U S A,* 2005, 13224-9.

Symons, H. J., Leffell, M. S., Rossiter, N. D., Zahurak, M., Jones, R. J. & Fuchs, E. J. (2010) Improved survival with inhibitory killer immunoglobulin receptor (KIR) gene mismatches and KIR haplotype B donors after nonmyeloablative, HLA-haploidentical bone marrow transplantation. *Biol Blood Marrow Transplant,* 16(4), 533-42.

Taner, S. B., Pando, M. J., Roberts, A., Schellekens, J., Marsh, S. G., Malmberg, K. J., Parham, P. & Brodsky, F. M. (2011) Interactions of NK cell receptor KIR3DL1*004 with chaperones and conformation-specific antibody reveal a functional folded state as well as predominant intracellular retention. *J Immunol,* 186(1), 62-72.

Thiruchelvam-Kyle, L., Hoelsbrekken, S. E., Saether, P. C., Bjornsen, E. G., Pende, D., Fossum, S., Daws, M. R. & Dissen, E. (2017) The activating human NK cell receptor KIR2DS2 recognizes a beta2-microglobulin-independent ligand on cancer cells. *J Immunol,* 198(7), 2556-67.

Traherne, J. A., Martin, M., Ward, R., Ohashi, M., Pellett, F., Gladman, D., Middleton, D., Carrington, M. & Trowsdale, J. (2010) Mechanisms of copy number variation and hybrid gene formation in the KIR immune gene complex. *Hum Mol Genet,* 19(5), 737-51.

Trompeter, H. I., Gomez-Lozano, N., Santourlidis, S., Eisermann, B., Wernet, P., Vilches, C. & Uhrberg, M. (2005) Three structurally and functionally divergent kinds of promoters regulate expression of clonally distributed killer cell Ig-like receptors (KIR), of KIR2DL4, and of KIR3DL3. *The Journal of Immunology,* 174(7), 4135-43.

Trundley, A. E., Hiby, S. E., Chang, C., Sharkey, A. M., Santourlidis, S., Uhrberg, M., Trowsdale, J. & Moffett, A. (2006) Molecular characterization of KIR3DL3. *Immunogenetics,* 57(12), 904-16.

Tu, M. M., Mahmoud, A. B. & Makrigiannis, A. P. (2016) Licensed and Unlicensed NK Cells: Differential Roles in Cancer and Viral Control. *Front Immunol,* 7, 166.

Uhrberg, M., Valiante, N. M., Shum, B. P., Shilling, H., Lienert-Weidenbach, K., Corliss, B., Tyan, D., Lanier, L. & Parham, P. (1997) Human diversity in killer cell inhibitory receptor genes. *Immunity,* 7, 753-63.

van Teijlingen, N. H., Holzemer, A., Korner, C., Garcia-Beltran, W. F., Schafer, J. L., Fadda, L., Suscovich, T. J., Brander, C., Carrington, M., Evans, D. T., van Baarle, D. & Altfeld, M. (2014) Sequence variations in HIV-1 p24 Gag-derived epitopes can alter binding of KIR2DL2 to HLA-C*03:04 and modulate primary natural killer cell function. *AIDS,* 28(10), 1399-408.

Vendelbosch, S., de Boer, M., Gouw, R. A., Ho, C. K., Geissler, J., Swelsen, W. T., Moorhouse, M. J., Lardy, N. M., Roos, D., van den Berg, T. K. & Kuijpers, T. W. (2013) Extensive variation in gene copy number at the killer immunoglobulin-like receptor locus in humans. *PLoS One,* 8(6), e67619.

Vierra-Green, C., Roe, D., Hou, L., Hurley, C. K., Rajalingam, R., Reed, E., Lebedeva, T., Yu, N., Stewart, M., Noreen, H., Hollenbach, J. A., Guethlein, L. A., Wang, T., Spellman, S. & Maiers, M. (2012) Allele-level haplotype frequencies and pairwise linkage disequilibrium for 14 KIR loci in 506 European-American individuals. *PLoS ONE,* 7(11), e47491.

Vierra-Green, C., Roe, D., Jayaraman, J., Trowsdale, J., Traherne, J., Kuang, R., Spellman, S. & Maiers, M. (2016) Estimating KIR haplotype frequencies on a cohort of 10,000 individuals: A comprehensive study on population variations, typing resolutions, and reference haplotypes. *PLoS One,* 11(10), e0163973.

Vilches, C., Gardiner, C. M. & Parham, P. (2000a) Gene structure and promoter variation of expressed and nonexpressed variants of the KIR2DL5 gene. *J Immunol,* 165(11), 6416-21.

Vilches, C., Pando, M. J. & Parham, P. (2000b) Genes encoding human killer-cell Ig-like receptors with D1 and D2 extracellular domains all contain untranslated pseudoexons encoding a third Ig-like domain. *Immunogenetics,* 51(8-9), 639-46.

Vilches, C. & Parham, P. (2002) KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol,* 20, 217-51.

Vivian, J. P., Duncan, R. C., Berry, R., O'Connor, G. M., Reid, H. H., Beddoe, T., Gras, S., Saunders, P. M., Olshina, M. A., Widjaja, J. M., Harpur, C. M., Lin, J., Maloveste, S. M., Price, D. A., Lafont, B. A., McVicar, D. W., Clements, C. S., Brooks, A. G. & Rossjohn, J. (2011) Killer cell immunoglobulin-like receptor 3DL1-mediated recognition of human leukocyte antigen B. *Nature,* 479(7373), 401-5.

Wagner, I., Schefzyk, D., Pruschke, J., Schofl, G., Schone, B., Gruber, N., Lang, K., Hofmann, J., Gnahm, C., Heyn, B., Marin, W. M., Dandekar, R., Hollenbach, J. A., Schetelig, J., Pingel, J., Norman, P. J., Sauter, J., Schmidt, A. H. & Lange, V. (2018) Allele-level KIR genotyping of more than a million samples: workflow, algorithm, and observations. *Front Immunol,* 9(2843), 1-15.

Wang, H., Grzywacz, B., Sukovich, D., McCullar, V., Cao, Q., Lee, A. B., Blazar, B. R., Cornfield, D. N., Miller, J. S. & Verneris, M. R. (2007) The unexpected effect of cyclosporin A on CD56+CD16- and CD56+CD16+ natural killer cell subpopulations. *Blood,* 110(5), 1530-9.

Wauquier, N., Padilla, C., Becquart, P., Leroy, E. & Vieillard, V. (2010) Association of KIR2DS1 and KIR2DS3 with fatal outcome in Ebola virus infection. *Immunogenetics,* 62(11-12), 767-71.

Wauquier, N., Petitdemange, C., Tarantino, N., Maucourant, C., Coomber, M., Lungay, V., Bangura, J., Debre, P. & Vieillard, V. (2019) HLA-C-restricted viral epitopes are associated with an escape mechanism from KIR2DL2(+) NK cells in Lassa virus infection. *EBioMedicine,* 40, 605-613.

Weisdorf, D., Cooley, S., Wang, T., Trachtenberg, E., Haagenson, M. D., Vierra-Green, C., Spellman, S., Spahn, A., Vogel, J., Kobusingye, H., Fehninger, T., Woolfrey, A., Devine, S., Ross, M., Waller, E. K., Sobecks, R., Parham, P., Guethlein, L. A., Marsh, S. G. E., Miller, J. & participating center writing, c. (2019) KIR donor selection: Feasibility in identifying better donors. *Biol Blood Marrow Transplant,* 25(1), e28-32.

Weisdorf, D., Cooley, S., Wang, T., Trachtenberg, E., Vierra-Green, C., Spellman, S., Sees, J. A., Spahn, A., Vogel, J., Fehniger, T. A., Woolfrey, A. E., Devine, S. M., Ross, M., Waller, E. K., Sobecks, R. M., McGuirk, J., Oran, B., Farag, S. S., Shore, T., Van Besien, K., Marsh, S. G. E., Guethlein, L. A., Parham, P. & Miller, J. S. (2020) KIR B donors improve the outcome for AML patients given reduced intensity conditioning and unrelated donor transplantation. *Blood Adv,* 4(4), 740-754.

Wilson, M. J., Torkar, M., Haude, A., Milne, S., Jones, T., Sheer, D., Beck, S. & Trowsdale, J. (2000) Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci U S A,* 97(9), 4778-83.

Winter, C. C., Gumperz, J. E., Parham, P., Long, E. O. & Wagtmann, N. (1998) Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *The Journal of Immunology,* 161, 571-77.

Winter, C. C. & Long, E. O. (1997) A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes. *J Immunol,* 158(9), 4026-4028.

Wroblewski, E. E., Parham, P. & Guethlein, L. A. (2019) Two to Tango: Co-evolution of Hominid Natural Killer Cell Receptors and MHC. *Front Immunol,* 10(177), 1-22.

Wu, S., Zeng, Y. J., Zhang, C., Deng, T. X., Xu, Y. Q. & Zhang, X. (2015) The role of the killer cell immunoglobulin-like receptor (KIR) "missing self" model in unrelated donor HSCT: a meta-analysis. *Transplant Proc,* 47(2), 558-65.

Wu, X., Yao, Y., Bao, X., Zhou, H., Tang, X., Han, Y., Ma, X., Liu, Y., Chen, J., Zhou, H., Jing, S., Gu, B., Xu, Y., Sun, A., He, J. & Wu, D. (2016) KIR2DS4 and its variant KIR1D are associated with acute graft-versus-host disease, cytomegalovirus, and overall survival after sibling-related HLA-matched transplantation in patients with donors with KIR gene haplotype A. *Biol Blood Marrow Transplant,* 22(2), 220-5.

Yahng, S. A., Jeon, Y. W., Yoon, J. H., Shin, S. H., Lee, S. E., Cho, B. S., Eom, K. S., Kim, Y. J., Lee, S., Min, C. K., Cho, S. G., Kim, D. W., Lee, J. W., Min, W. S. & Kim, H. J. (2016) Negative impact of unidirectional host-versus-graft killer cell immunoglobulin-like receptor ligand mismatch on transplantation outcomes after unmanipulated haploidentical peripheral blood stem cell transplantation for acute myeloid leukemia. *Biol Blood Marrow Transplant,* 22(2), 316-23.

Zhou, H., Bao, X., Wu, X., Tang, X., Wang, M., Wu, D. & He, J. (2014) Donor selection for KIR B haplotype of the centromeric motifs can improve the outcome after HLA-identical sibling hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant,* 20, 98-105.

Chapter 2 | Materials & Methods

General equipment and plastic consumables were obtained from: pipettes and tips [manufactured by Mettler-Toledo Rainin, United States, distributed by Anachem, United Kingdom], bijoux tubes, microtubes and eppendorf tubes [Alpha Laboratories, United Kingdom], 96-well PCR plates and pastettes [Scientific Laboratory Supplies, United Kingdom], 96-well deep well storage plates [Thermo Fisher Scientific, United States], reservoirs [Integra Biosciences, United States].

The following techniques were used throughout the thesis.

2.1 DNA extraction & quantification

Residual DNA was available for all transplants and recipients tested. Volunteer DNA samples were extracted from whole blood.

A 350 µL whole blood sample from the individual was loaded onto an EZ1 Advanced XL automated DNA extraction instrument [Qiagen, Germany] for 200 µL final solution volume, using 'EZ1 DNA Blood 350 µL kit' reagent cartridges [manufactured by Qiagen, Germany, distributed by Alpha Biotech, United Kingdom]. The DNA extraction product was placed on a magnet for 20 s, and the supernatant DNA solution collected for testing.

DNA samples were quantified by two methods: spectrophotometry and fluorometry.

A 2 µL sample was quantified by a Nanodrop One^{c} spectrophotometer [Thermo Fisher Scientific, United States]. The spectrophotometer baseline was set using molecular-grade water. DNA concentration (ng/µL), and 260nm/280nm absorbance ratio from each sample were collected.

DNA concentration was also measured by a Qubit 3 fluorometer [Invitrogen, United States], using the dsDNA broad range assay kit [Invitrogen, United States]. A reagent mastermix was constituted using 199 µL of Qubit buffer [Invitrogen, United States] and 1 µL of Qubit reagent [Invitrogen, United States] per sample plus two standards. Two standards (Standard 1 and Standard 2 [Invitrogen, United States]) were then made up in a fluometry eppendorf tube, with 190 µL of reagent mastermix 10 µL of the relevant Qubit Standard. Sample tests were set up using 198 µL of Qubit buffer/reagent mastermix and 2 µL of DNA sample. The standards and samples were briefly vortexed at setting 7 of a Vortex-Genie 2 vortex [Scientific Industries, United States], and incubated at ambient room temperature in a dark cupboard for 2 min. Following incubation, the standards were read on the fluorometer to establish a standard curve for measurements, using the broad range dsDNA programme. Each DNA sample concentration (ng/µL) was then read on the fluorometer.

DNA concentration data from fluorometry was used for calculating required DNA volumes in all methods due to its high sensitivity and specificity. The spectrophotometry results were used to assess the quality of DNA prior to testing, with a 260nm/280nm absorbance ratio of approximately 1.8 accepted as high level of DNA purity. Measurements outside of range did not preclude the sample progressing to testing, but could be used as a metric of DNA quality in the event of a test failure.

2.2 KIR presence/absence genotyping: sequence-specific oligonucleotide testing for presence/absence of KIR genes

Sequence-specific oligonucleotide (PCR-SSO) genotyping of KIR genes was carried out using the LABType KIR SSO kit [manufactured by OneLambda, United States, all products distributed by V.H.Bio, United Kingdom], following local protocols,

modified from the manufacturer's recommended protocol. Briefly, this was as follows:

2.2.1 PCR Amplification

A PCR mix was set up in a 96-well PCR tray with each LABType KIR primer set, made of 2 µL of sample DNA (native concentration), 6.9 µL of LABType D-mix, 2 µL of the respective LABType amplification primer (each test consists of three primer sets: KIR G1/2/3) [all LABType reagents OneLambda, United States], 0.05 µL of Taq polymerase [Bioline, United Kingdom, distributed by V.H.Bio, United Kingdom], and 0.05 µL of molecular grade water. Each KIR test consisted of three sets of primers, KIRG1, 2, and 3, each treated as an independent test.

The tests were briefly centrifuged up to 95 RCF in a Universal 320 centrifuge [Hettich, Germany] to ensure contents were at the bottom of each well, and were then amplified on a Veriti thermal cyler [Applied Biosystems, United States] using the PCR programme listed in Table 4 (all subsequent thermal cycler steps use a thermal cycler).

Step	Temperature	Time	Number of cycles
Initial Denaturation	96 °C	3 min	-
Denaturation	96 °C	20 sec	5 cycles (3 steps)
Annealing	60 °C	20 sec	
Elongation	72 °C	20 sec	
Denaturation	96 °C	10 sec	5 cycles (3 steps)
Annealing	60 °C	15 sec	
Elongation	72 °C	20 sec	
Final Elongation	72 °C	1 min	1 cycle
Cooling	4 °C	Infinity	-

Table 4 - PCR cycling protocol for KIR LABType amplification.

2.2.2 Denaturation & Neutralisation

2.5 µL of amplified PCR product was transferred to a clean 96-well tray, and 1.25 µL of LABType denaturation buffer [OneLambda, United States] was added to each test

well. The plate was then covered and centrifuged to ensure the contents are mixed. The test tray was incubated at 21 °C for 10 min.

Following incubation, 2.50 µL of LABType denaturation buffer [OneLambda, United States] was added to each test well, and the plate was then covered and centrifuged to ensure mixing of the contents. The test mix was assessed for a pink to yellow colour change, indicative of a neutralised solution. 1.00 µL of LABType neutralisation buffer [OneLambda, United States] was added to any test well where no colour change was observed, and vortexed to mix solution until colour change was achieved.

2.2.3 Hybridisation

The following steps took place with the PCR tray on a cooler tray (out of -10°C storage). 19 µL of hybridisation mixture, made up of 17 µL of LABType Hybridisation Buffer and 2 µL of the relevant KIR G1/2/3 LABType bead mixture [OneLambda, United States], was added to each test well. The PCR tray was then covered and incubated at 60 °C for 15 min on a thermal cycler.

Two wash cycles were carried out, each cycle consisting of: 50 µL of LABType wash buffer [OneLambda, United States] was added, the test tray was centrifuged for 5 min at 160 RCF in a Universal 320 centrifuge, and the wash buffer supernatent removed by flicking into a sink. The test tray was then vortexed on a Vortex-Genie 2 vortex to dislodge the pellet of beads from the bottom of the test wells.

2.2.4 Fluorescent Labelling

25 µL of SAPE solution, made up of 0.25 µL LABType SAPE Stock and 24.75 µL of LABType SAPE Buffer [OneLambda, United States], was added to each test well, and the test tray was incubated for 5 min at 60 °C on a thermal cycler. One wash cycle

was completed (as previously described in Chapter 2.2.3), and 70 µL LABType wash buffer was added to each test well, and each test mixture was mixed gently by pipette.

2.2.5 Loading onto LABScan 3D

The test tray was loaded onto a LABScan 3D Luminex instrument [OneLambda, United States] for reading of results.

2.2.6 Analysis

Analysis was carried out using HLA Fusion 3.0 [OneLambda, United States], and reference sequences from the 2.6.1 IMGT/KIR database release (Robinson et al., 2010). The following analysis criteria were used:

- >1,000 bead count for exon positive control amplification fluorescence intensity
- >50 bead count for each bead identity

The beadset thresholds (set by the manufacturer) were compared against the bead mean fluorescence intensities for positive/negative results.

In tests where a borderline result (either side of the threshold) would affect the KIR diplotype call (the presence/absence of a KIR gene), verification testing by real-time PCR (qPCR) was carried out (section 2.3). All tests demonstrating less common KIR diplotypes (≤3 samples with the same diplotype within the cohort) were also tested by qPCR to verify the result.

2.3 KIR presence/absence genotyping: Real-time polermase chain reaction testing for presence/absence of KIR genes

KIR genotyping by real-time PCR (qPCR) was carried out using the OneLambda LinkSêq KIR typing kit [OneLambda, United States], following a local protocol adapted from the manufacturer's recommended protocol (Anonymous, 2018b). The following describes the local protocol used.

2.3.1 Test Set-Up

The PCR mix (minus sample DNA) was constituted, made of the pre-constituted vial of PCR mix, 9 µL Taq Polymerase [OneLambda, United States], and a variable volume of molecular grade water (224 µL minus the sample volume of DNA to be added). The solution was gently mixed by inversion. 10µL of this mixture was added to the no template negative control well of the LinkSeq test tray. A volume of sample DNA equal to 370 ng of DNA was added to the PCR mixture, and the solution gently mixed by inversion. 10µL was added to all remaining test wells.

Step	Temperature	Time	Number of cycles
Initial Denaturation	95 °C	2 min	1 cycle
Denaturation	95 °C	15 sec	36 cycles (3 steps)
Annealing & Elongation	64 °C	60 sec	
Denaturation	95 °C	15 sec	1 cycle
Annealing & Elongation	65 °C	30 sec	1 cycle
Dissociation (Melt Curve)	65-95 °C	--	1 cycle
	95 °C	15 sec	
Cooling	4 °C	Infinity	--

Table 5 – Real time PCR cycling protocol for KIR LinkSeq kit.

2.3.2 Loading onto LightCycler real time PCR instrument

The PCR tray was sealed, vortexed to mix ona Vortex-Genie 2 vortex, and centrifuged at 95 RCF for 1 minute to bring the mixture to the bottom of each test well. The PCR

tray was loaded onto the LightCycler® 480 II qPCR instrument [Roche Life Science, Switzerland], and run for the relevant qPCR profile described in Table 5.

2.3.3 Analysis

The LightCycler® 480 II melt curve data was imported into SureTyper™ v5 for analysis [OneLambda, United States], using reference sequences from the 2.6.1 IMGT/KIR database release (Robinson et al., 2010). . Each automated test call was checked for PCR peak position in the established negative and positive peak regions, including the no template negative control. Tests with any number of failed wells were repeated using LABType SSO testing for verification.

2.4 KIR allelic definition: next generation sequencing of select KIR genes

Step	Temperature	Time	Number of cycles
Initial Denaturation	94 °C	2 min	-
Denaturation	94 °C	20 sec	30 cycles (2 steps)
Annealing/Elongation	60 °C	8 min	
Final Elongation	60 °C	10 min	-
Cooling	15 °C	Infinity	-

Table 6 – PCR cycling protocol for KIR amplification.

The following protocol was used for all KIR gene sequencing, using experimental NGSgo®-AmpX KIR kits [GenDx, Netherlands], following manufacturer's recommended protocol (Anonymous, 2017a; Anonymous, 2018a). At the time of testing, a limited number of KIR gene sequencing primers were available for genotyping from the manufacturer. All KIR genes with available primers were sequenced: KIR2DL1, 2DL2, 2DL3, 2DL4, 3DL2, 3DL3, and 3DL1S1 (divided into separate KIR3DL1 and KIR3DS1 primer pools due to close homology).

2.4.1 PCR Amplification

Each KIR gene was amplified separately using the PCR protocol listed in Table 6. The reaction mixture was made of (per test well) 2 µL DNA Sample (20 ng/µL), 2 µL KIR gene-specific primer, 3.5 µL nuclease-free water, 2.5 µL LongMix PCR master mix [GenDx, Netherlands]. For purposes of testing, KIR3DL1 and 3DS1 were treated as separate reactions. All reactions were set up on a cooling block. The KIR3DS1 amplification tests was omitted for samples shown to be KIR3DS1 negative by presence/absence genotyping (SSO or qPCR genotyping).

Following amplification, the PCR products were pooled per sample in a PCR plate, as described in Table 6.

Pool	Component	Volume (per sample)
Pool 1	KIR2DL1 PCR Product	2.3 µL
	KIR3DL1 PCR Product	2.1 µL
	Molecular Grade Water	19.8 µL
Pool 2	KIR2DL3 PCR Product	2.8 µL
	KIR2DL4 PCR Product	1.0 µL
	KIR3DL3 PCR Product	1.0 µL
	Molecular Grade Water	19.4 µL
Pool 3	KIR2DL2 PCR Product	2.4 µL
	KIR3DL2 PCR Product	2.6 µL
	Molecular Grade Water	19.2 µL
Pool 4	KIR3DS1 PCR Product	5.0 µL
	Molecular Grade Water	19.2 µL

Table 7 – Pooling of PCR products for library preparation.

2.4.2 Monitoring of PCR product

Following amplification, successful amplification was monitored by agarose gel electrophoresis. The agarose gel electrophoresis was prepared with 1% molecular biology grade agarose gel [Severn Biotech, United Kingdom] in 1x tris borate electrophoresis (TBE) buffer (0.089M tris, 0.089M boric acid, 0.002M EDTA, pH8.3)

[Severn Biotech, United Kingdom], loaded with a mix of 2 µL of PCR product and 4 µL of DNA loading buffer [Bioline, United Kingdom]. Gels were run in a horizontal electrophoresis MultiSub Maxi tank (20x10cm gel) [Biocomdirect, United Kingdom] filled with 1x TBE buffer, and visualised using SafeView nucleic acid stain (50 µL per litre of TBE buffer, added to the stock TBE buffer) [NBS Biologicals, United Kingdom]. The gel was run for 1 hour at 140 volts and 120 milliamps on a Power 300 electrophoresis power supply [Thermo Fisher Scientific, United States]. Gels were visualised using an E-box gel imager [Vilber Lourmat, France]. A distinct band was interpreted as a successful amplification. Expected sizes of bands are described in Table 8. Where no indication of a band was present, this data was cross-referenced with PCR-SSO/qPCR data to establish if this was a true negative for the respective gene or a failed test. All PCR products proceeded to testing, with monitor gels used as part of the evaluation of any test failures.

Gene	Band Size (kb)	Gene	Band Size (kb)
KIR2DL1	14.9	KIR3DL1	14.8
KIR2DL2		KIR3DS1	15.1
KIR2DL3		KIR3DL2	17.1
KIR2DL4	11.1	KIR3DL3	13.6

Table 8 – Expected DNA band sizes in monitor agarose gel electrophoresis.

All following reactions were set up on cooling block, until stated otherwise.

2.4.3.2 Fragmentation

A fragmentation mix was constituted, made of (per test well) 2 µL NGSgo®-LibrX fragmentase buffer, 3.25 µL NGSgo®-LibrX end prep buffer, 1.5 µL NGSgo-LibrX fragmentase enzyme, and 1.5 µL NGSgo®-LibrX end prep enzyme [GenDx, Netherlands], and added to the 24.2 µL of PCR product/nuclease-free water mixture. The test tray was then briefly vortexed on a Vortex-Genie 2 vortex to mix the tests, and centrifuged at 95 RCF for 30 s in a Universal 320 centrifuge. The test tray was

then placed in a thermal cycler, using the fragmentation protocol: 25 °C for 20 min, 70 °C for 10 min, and then 15 °C holding temperature until tray is removed.

2.4.3.3 Ligation of adapter sequences

During fragmentation, the master mix for ligation was constituted, made of (per test well) 7.5 µL NGSgo-LibrX ligase mix, 0.5 µL NGSgo-LibrX ligation enhancer, 0.25 µL NGSgo-LibrX adapter for Illumina [GenDx, Netherlands], and 1 µL Nuclease-free water, and stored on ice. Following fragmentation, the test tray was centrifuged at 95 RCF for 30 s in a Universal 320 centrifuge. The ligation master mix was added directly to each test well, before being briefly vortexed on a Vortex-genie 2 vortex and centrifuged to 95 RCF for 30 s in a Universal 320 centrifuge. The test trays were placed on a thermal cycler using the ligation protocol: 20 °C for 15 min (adapter ligation), and a 15 °C cooling step held until tray was removed.

2.4.3.4 Cleaning I

Following adapter ligation, the test trays were centrifuged at 95 RCF for 30 s in a Universal 320 centrifuge. All following testing was at ambient room temperature (no cooling blocks used). The assays were transferred from a PCR tray to a deep-welled tray, and 18.8 µL of AMPure XP magnetic SPRI beads [Beckman Coulter, United States] added to each test. The tests were then vortexed for 10 s on a Vortex-Genie 2 vortex to mix, and then the plates were incubated at room temperature for 15 min on a Bioshake iQ thermoshaker at 1,500 rpm [Quantifoil Instruments, Germany] (all subsequent thermoshaker steps described use a Bioshake iQ thermoshaker). Following this, plates were centrifuged at 95 RCF for 30 min in a Universal 320 centrifuge to collect the tests at the bottom of the wells, whilst retaining the beads in suspension, and then further incubated for 5 min on a magnetic stand-96 [Invitrogen,

United States] to completely separate the magnetic beads from the mixture (lid removed prior to incubation to minimise disturbance of tests).

After the incubation, the tests were inspected to confirm the mixture is clear (i.e. beads removed from mixture). Supernatant was removed by pipetting. A total of three ethanol washes were applied, with each wash cycle consisting of: 200 µL of freshly constituted 80% ethanol (solution of pure ethyl alcohol [Sigma-Aldrich, United States] and molecular grade water at 4 ml : 1 ml ratio), was added to each test well, the tray was incubated for 30 s on a magnetic stand-96, and the supernatant was removed by pipetting.

Following three washes, all residual supernatant was removed by pipette. The tests were then air-dried for 5 min to allow residual ethanol to evaporate. Following air-drying, the tray was taken off the magnet, and 12.5 µL of Tris-EDTA (TE) buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0) [Invitrogen, United States] was added to elute the PCR products from the SPRI beads. The test tray was vortexed for 30 s to ensure the beads were in solution, and incubated at room temperature on a thermoshaker for 2 min at 1,500 rpm. The trays were then centrifuged at 95 RCF for 30 s in a Universal 320 centrifuge, and incubated on a magnetic stand-96 for 5 min. After incubation, the tests were inspected to confirm the mixture is clear. 10 µL of eluate was transferred into a PCR tray.

2.3.3.5 Indexing

12.5 µL of NGSgo®-Indx index [GenDx, Netherlands] were added to the eluate. The test tray was vortexed on a Vortex-Genie 2 vortex, then centrifuged at 95 RCF for 30 s in a Universal 320 centrifuge in preparation for being placed on a thermal cycler with the PCR protocol listed in Table 9.

Step	Temperature	Time	Number of cycles
Initial Denaturation	98 °C	30 sec	1 cycle
Denaturation	98 °C	10 sec	10 cycles
Annealing	65 °C	30 sec	
Elongation	72 °C	30 sec	
Final Elongation	72 °C	5 min	1 cycle
Cooling	15 °C	Hold (Until tray is removed)	-

Table 9 – PCR cycling protocol for KIR indexing.

Following indexing, the tests were transferred to a deep-welled tray. 15 µL of AMPure XP magnetic SPRI beads were added to each test. The test tray was vortexed for 10 s, centrifuged briefly, and incubated for 5 min at room temperature on a thermoshaker at 1,500 rpm. The tray was centrifuged at 95 RCF for 30 s on a Universal 320 centrifuge, and then the test tray was incubated on a magnetic stand-96 for 5 min at ambient room temperature. The tests were inspected for confirmation that the solution was clear. A total of two ethanol wash cycles were applied. After the wash cycles, all residual supernatant was removed by pipette. The tests were then air-dried for 5 min as previously described (Chapter 2.4.3.4).

2.4.3.6 Cleaning II

Following air-drying, the tray was taken off the magnet, and 16.5 µL of Tris-EDTA (TE) buffer (10mM Tris-HCl, 0.1mM EDTA) [Invitrogen, United States] was added to elute the PCR products from the SPRI beads. The test tray was vortexed for 30 s to ensure the beads were in solution, and incubated at room temperature on a thermoshaker for 2 min at 1,500 rpm. The trays were centrifuged at 95 RCF for 30 s in a Universal 320 centrifuge, and incubated on a magnetic stand-96 for 5 min at ambient room temperature. After incubation, the tests were inspected to confirm the mixture is clear. 14 µL of eluate was transferred into a PCR tray.

2.4.3.7 Pooling II

The eluate for each test was pooled into a single library pool, ensuring the volume was proportional to the number of loci contained within each test: 3 µL for one locus pools, 6 µL for two loci pools, 9 µL for three loci pools.

2.4.4 Library pool preparation for loading onto sequencer instrument

Library pool preparation was carried out in line with the manufacturer's recommended protocol for use of MiSeq next generation sequencer instrument [illumina, United States].

The DNA concentration was measured using the QuBit instrument, and then diluted to 4 ng/µL with TE buffer (10mM Tris-HCl, 0.1mM EDTA). For denaturation of the DNA, 10 µL 0.2M sodium hydroxide (NaOH) [Fluka Analytical, Switzerland] was added to 10 µL of the final diluted library pool. The solution was vortexed briefly, centrifuged briefly, and incubated at room temperature for five min. To halt denaturation, 980 µL of hybridisation solution was added to the library/NaOH solution. 600 µL of the library solution was loaded onto an illumina MiSeq v2 (300 cycle) cartridge [illumina, United States], which was then loaded into the illumina MiSeq instrument for sequencing.

2.4.5 Analysis

All KIR NGS analysis was carried out using NGSengine v2.16 [GenDx, Netherlands], and reference sequences from the 2.8.0 IMGT/KIR database release (Robinson et al., 2010). In accordance with manufacturer's guidance, all exons were analysed, with regions excluded from analysis listed in Table 10.

Gene	Excluded Sequence Regions	Gene	Excluded Sequence Regions
KIR2DL1	35 – 998 5,195 – 5,214 6,380 – 7,325	KIR3DL1	35 – 1,033 6,531 – 6,551
KIR2DL2	35 – 942 5,110 – 5135 5,950 – 5,975 6,295 – 6,445	KIR3DL2	35 – 744 4,586 – 4,598 12,328 – 1,2349:1 12,521 – 12,537
KIR2DL3	35 – 942 5,113 –5,133 5,967 – 5,980 6,308 – 6,417	KIR3DL3	35 – 711 7,720 – 7,731:1
KIR2DL4	449 – 533 2217 – 2234	KIR3DS1	35 –1,033

Table 10 – Excluded sequence regions for each KIR gene, in accordance with manufacturer's guidelines.

The NGS was analysed using the following quality metrics:

- Minimum exonic depth of coverage: 90 reads for heterozygous loci, 45 for homozygous loci.
- Maximum Noise threshold: 14%
- Minimum heterozygous allele balance: 20%
- Delta signal to noise percentage (differential between highest noise percentage and lowest heterozygous position): 10%

The overall quality was reviewed for the above quality metrics. All heterozygous positions within each sequenced exon were reviewed for allele depth. Where ambiguities were present (e.g. due to phasing), the closest intronic match was selected as the allele call.

2.5 HLA Allelic Definition: Next generation sequencing of HLA genes

NGS of HLA class I & II was carried out using Trusight HLA v2 NGS kits [manufactured by illumina, United States, distributed by CareDx, United States],

following the manufacturer's recommended protocol (Anonymous, 2017b). Although HLA class I & II data was collected as part of this kit, only HLA class I data was used in this thesis.

Tray A Protocol				Tray B Protocol			
Step	Temperature	Time	Number of cycles	Step	Temperature	Time	Number of cycles
Initial Denaturation	94 °C	3 min	1 cycle	Initial Denaturation	94 °C	3 min	1 cycle
Denaturation	94 °C	30 sec	30 cycles	Denaturation	94 °C	30 sec	10 cycles
				Annealing	55 °C	2 min	
Annealing	60 °C	2 min		Elongation	72 °C	15 min	
				Denaturation	94 °C	30 sec	20 cycles
Elongation	68 °C	15 min		Annealing	60 °C	2 min	
				Elongation	72 °C	15 min	
Final Elongation	68 °C	10 min	1 cycle	Final Elongation	72 °C	10 min	1 cycle
Cooling	4 °C	Hold (Until tray is removed)	-	Cooling	4 °C	Hold (Until tray is removed)	-

Table 11 – PCR cycling protocol for amplification of HLA genes.

Tray A protocol was used for amplification of HLA-A, B, C, DRB1, DQA1, DPA1, and DPB1. Tray B protocol was used for amplification of HLA-DQB1 only.

2.5.1 PCR Amplification

Each DNA sample was diluted to 10ng/µL. Samples below 10ng/µL were tested neat. 5 µL of DNA was added to each test well. Eight test wells were used for each sample: HLA-A, B, C, DRB1/3/4/5, DQA1, DPA1, and DPB1 on tray A, and DQB1 on tray B. 5 µL of HLA locus-specific primer [manufactured by illumina, United States, distributed by CareDx, United States] was added to its relevant test well. The PCR mix was constituted, made of (per test well) 25 µL of HLA PCR Mix, 2 µL of MasterAmp Extra-Long DNA Polymerase, 13 µL of PCR grade water [manufactured by illumina, United States, distributed by CareDx, United States]. 40 µL of PCR mix was added to each test well.

The trays were sealed, vortexed to mix, and centrifuged at 280 G for 2 min to settle the mix at the bottom of the test trays. The test trays were then placed in thermal cyclers for the respective cycle protocols for tray A and B (Table 11).

2.5.2 Library Preparation

2.5.2.1 Ethanol Wash

Following amplification, 45 µL of PCR product was transferred to new 96-well trays. 31.5 µL of TruSight sample purification beads (SPB) [manufactured by illumina, United States, distributed by CareDx, United States] were added to each test well. The trays were covered and incubated on a thermoshaker at 1800 rpm for 2 min, and then 2 min further without shaking. They were then placed on a magnetic stand-96 and incubated for 2 min, until the solution was clear and the SPB formed a pellet. The supernatant was discarded by pipette.

One ethanol wash cycle was carried out: 200 µL of freshly constituted 80% ethanol (pure ethyl alcohol/distilled water solution) was added to each test well, the tray was incubated for 30 s on a magnetic stand-96, and the supernatant was removed by pipetting. Following the wash step, all residual ethanol was removed by pipette and subsequent 5-minute air dry.

30 µL of resuspension buffer (RSB) [manufactured by illumina, United States, distributed by CareDx, United States] was added to each test well. The trays were covered and incubated for 2 min on a thermoshaker at 1800 rpm, until the SPB were evenly mixed in the RSB. After a further 2 min incubation without shaking, the trays were placed on a magnetic stand-96 and incubated for 2 min, until the solution was clear and a pellet was beads was formed. 20 µL of the supernatant was transferred to

new 96-well trays for further library preparation, and 5 µL was transferred into a 96-well tray for monitoring of PCR product.

2.5.3 Monitoring of PCR Product

5 µL of the cleaned PCR product were allocated into a 96-well PCR tray, and mixed by pipette with 5 µL of DNA loading buffer. The PCR product/loading buffer was then run on the gel electrophoresis system (described in Chapter 2.4.2), using a 2% agarose gel in 1x TBE buffer (0.089M tris, 0.089M boric acid, 0.002M EDTA, pH8.3). Following electrophoresis, the gel was visualised using an E-box gel imager. Presence of a clear band on the gel image was indicative of successful amplification, with absence of a clear band suggesting potentially failed amplification.

All samples were taken forwarded for library preparation, regardless of presence/absence monitor gel bands. The monitor gel image was used in cases of failed tests to diagnose possible issues with the respective sample.

2.5.4 Library Preparation Continued

2.5.4.1 Normalisation

The normalisation bead solution mixture was constituted, made of (per sample) 366.7 µL of TruSight Library Normalisation Additives, and 33.3 µL TruSight Library Normalisation Beads [manufactured by illumina, United States, distributed by CareDx, United States], and then 45 µL of this normalisation bead solution was added to each test well. The trays were sealed, and incubated at ambient room temperature for 30 min on a thermoshaker at 1,800 rpm. The test trays were then incubated on a magnetic stand-96 for 2 min, until the solution was clear and the SPB formed a pellet. All supernatant was removed by pipette, and 45 µL of RSB was added to each test

well, trays were then incubated for 5 min on a thermoshaker at 1,800 rpm. The trays were then each incubated on a magnetic stand-96 for 2 min, until the solution was clear and the SPB formed a pellet. All supernatant was removed by pipette.

2.5.4.2 Tagmentation of Amplicons

The trays were removed from the stands, and 40 µL of TruSight HLA tagmentation buffer [manufactured by illumina, United States, distributed by CareDx, United States] was added to each test well. The trays were sealed and incubated for 5 min on a thermoshaker at 1,800 rpm. 10 µL of HLA tagmentation mix [manufactured by illumina, United States, distributed by CareDx, United States] was added to each test well, and mixed by pipette. The trays were immediately incubated at 58 °C for 12 min in a thermal cycler, and subsequently incubated for 2 min, until the solution was clear and the SPB formed a pellet. 50 µL of supernatant were transferred to a new 96-well tray.

2.5.4.3 Library Pooling

The tagmented amplicons of each HLA locus amplicon were pooled together in a new 96-well tray, such that the library pool for each sample was held in a single test well: 10 µL each of HLA-A, B, C, DQA1, DQB1, DPA1, and DPB1 amplicons, and 20 µL of DRB1/3/4/5 amplicons.

2.5.4.4 Ethanol Wash

A volume of 63 µL SPB was added to each test well. The trays were incubated at ambient room temperature for 2 min on a thermoshaker at 1,800 rpm, 2 min without shaking, and 5 min on a magnetic stand-96, until the solution was clear and the SPB formed a pellet.

Two ethanol wash cycles were carried out, and then the trays were air dried for 5 min, after which 22.5 µL RSB was added to each test. The trays were incubated at ambient room temperature for 2 min on a thermoshaker at 1800 rpm, and a further 2 min without shaking, followed by a 2 min incubation on a magnetic stand-96, until the solution was clear and the SPB formed a pellet. 20 µL of the supernatant was transferred to a new tray for further testing.

Step	Temperature	Time	Number of cycles
Initial Annealing & Elongation	72 °C	3 min	1 cycle
Denaturation	98 °C	30 sec	
Denaturation	98 °C	10 sec	10 cycles
Annealing	60 °C	30 sec	
Elongation	72 °C	5 min	
Final Elongation	72 °C	5 min	1 cycle
Cooling	10 °C	Hold (Until tray is removed)	-

Table 12 – PCR cycling protocol for indexing.

2.5.4.5 Indexing

The TruSeq index adapters [manufactured by illumina, United States, distributed by CareDx, United States] were arranged in two sets, which together form the unique index sequence for each sample: 5 µL of the respective set 1 index were added to each test well down the columns of the test tray, and 5 µL of the respective set 2 index were added across the rows. A 20 µL of library amplification mix were added to each test, the tray was centrifuged at 160 RCF for 2 min in a Universal 320 centrifuge. The trays were loaded onto a thermal cycler, using the protocol described in Table 12.

2.5.4.6 Ethanol Wash & Final Pooling

45 µL of the PCR product were transferred to a new 96-well plate. 31.5 µL of SPB were added to each test well. The tray was sealed, and incubated at ambient room temperature for 2 min on a thermoshaker at 1800 rpm, and then incubated for a further

2 min without shaking. The tray was placed on a magnetic stand-96 and incubated for 2 min, until the solution was clear and the SPB formed a pellet.

Two ethanol wash cycles were carried out. The tray was then air dried for 5 min. 32.5 µL of RSB was added to each test well. The tray was covered, and incubated for 2 min on a shaker at 1,800 rpm, and incubated for a further 2 min without shaking. The tray was placed on a magnetic stand-96 for 2 min, until the solution was clear and the SPB formed a pellet.

30 µL of supernatant was transferred to a new 96-well tray. 7 µL of each test well were pooled together for the final library pool. The DNA concentration of library pool was measured by a Qubit fluorometer (as described in Chapter 2.1), and was then diluted to 1.5 ng/µL with RSB.

2.5.5 Loading onto the MiSeq

10 µL of 0.1 M NaOH was added to 10 µL of the diluted library pool, and incubated for 5 min. At the end of incubation, 980 µL of hybridisation buffer [manufactured by illumina, United States, distributed by CareDx, United States] was immediately added. 600 µL of the denatured library pool were loaded onto an illumina MiSeq v2 (300 cycle) cartridge [illumina, United States], which was then loaded into the illumina MiSeq instrument for sequencing.

2.5.6 Analysis

All HLA NGS analysis was carried out using Assign TruSight v2.1.0.943, and reference sequences from the 3.37 IMGT/HLA database release (Robinson et al., 2010).

The NGS was analysed using the following quality metrics:

- Minimum exonic depth of coverage: 100 reads for heterozygous loci, 50 for homozygous loci.
- Quality score of 30 (Q30) (Anonymous, 2011): >75%
- Minimum heterozygous allele balance: 30% (exception: 20% for HLA-B*27, and HLA-DQB1*06)
- PCR Crossover: <15%

The overall quality was reviewed for the above quality metrics. All positions flagged by the software for manual interpretation were analysed. Where ambiguities were present (e.g. due to phasing), the most common combination of alleles were selected as the primary call.

Chapter 2 References

Anonymous. (2011) Quality scores for next-generation sequencing (illumina).

Anonymous. (2017a) GenDx NGSgo Workflow: Compatible with Illumina. Instructions for use. Edition 4 2017/06.

Anonymous. (2017b) TruSight HLA v2 sequencing panel reference guide (Illumina) v1.

Anonymous. (2018a) GenDx KIR amplification edition 0.1, 2018/06 [PROTOTYPE].

Anonymous. (2018b) LABType SSO typing tests: Product insert Rev 29.

Robinson, J., Mistry, K., McWilliam, H., Lopez, R. & Marsh, S. G. (2010) IPD - The Immuno Polymorphism Database. *Nucleic Acids Res,* 38(Database issue), D863-9.

Chapter 3 | Killer-cell immunoglobulin-like receptor and ligand genetic diversity in a single transplant centre cohort

Summary

The killer-cell immunoglobulin-like receptor (KIR) gene cluster comprises an assortment of highly polymorphic genes involved in the cytotoxicity and immunomodulation activities of natural killer (NK) cells and some subsets of T lymphocytes. With the advent of next generation sequencing (NGS), it is now viable for clinical laboratories to explore the polymorphism of KIR genes within their patient and donor population. This study aims to explore the gene content and allelic polymorphism observed in a single transplantation centre (n=281) to provide insight into the genetic diversity of both KIR and genes of their cognate ligands (HLA class I) encountered in the United Kingdom. Twenty-five unique KIR diplotype profiles were defined within the cohort, with 32.0% possessing KIR A/A 1 diplotypes, and 68.0% possessing variants of KIR B/x diplotypes. Seven KIR genes were sequenced by amplicon-based short read (ABSR) NGS: KIR2DL1, 2DL2, 2DL3, 2DL4, 3DL1S1, 3DL2, and 3DL3. This identified extensive polymorphism, particularly within KIR3DL2 and 3DL3, where 34 and 61 alleles were defined within the cohort respectively. Novel alleles were identified in all genes sequenced, with the exception of KIR2DL2, with many of these alleles verified by hybridisation-based targeted enrichment (HBTE) NGS. Diversity within the KIR ligands was also observed, with similar HLA-C epitope frequencies for C1/2 heterozygous (43.8%) and C1/1 homozygous (42.3%) individuals, while a sizable minority of 13.9% were C2/2 homozygous. 69.8% of the cohort was positive for the HLA-Bw4 epitope, with the most common forms of Bw4 (residue pos77-83) being N--TALR (positive in 35.6% of the cohort) and N--IALR (34.5% of the cohort). The diversity observed in this study will provide population data to inform and aid the construction of clinical algorithms involving KIR assessments, such as the selection haematopoietic progenitor cell donors.

3.1 Introduction

The killer-cell immunoglobulin-like receptor (KIR) gene cluster, located on human chromosome 19q13.4, may be the most polymorphic gene system in the human genome, forecast to even surpass that of the major histocompatibility complex (MHC) region, based onto the rapid rate of discovery of novel KIR alleles being registered with the European Bioinformatics Institute (EMBL-EBI) (Misra et al., 2018). Despite the rapid expansion of published alleles, the population dynamics of KIR compared to those of other genes of the immune system, particularly the human leukocyte antigen (HLA) genes, remain relatively unknown.

KIR genes encode glycoprotein surface-bound receptors of Natural Killer (NK) cells, and subsets of other lymphocytes, such as TcR$\alpha\beta^{+}$ invariant NK T (iNKT) lymphocytes. These receptors integral to the immunoregulation of NK cells, particularly in immunosurveillance of viral infection and malignancy (Huard and Karlsson, 2000; Bjorkstrom et al., 2012; Lisnic et al., 2015; Barbarin et al., 2017; Krasnova et al., 2017). A majority of KIR glycoproteins regulate the NK cell's cytotoxic activity through inhibitory or activating signalling upon interaction with their respective ligands (Campbell and Hasegawa, 2013; Ivarsson et al., 2014; Nash et al., 2014). Once activating signals exceed those of inhibitory signals, the NK cell will be activated to exert its cytotoxic activity upon the target cell via degranulation of perforin and granzymes.

In contrast to the immunoregulation of other KIR gene products, KIR2DL4 and potentially KIR3DL3 encode surface-bound receptors that are vital for decidual Natural Killer cell activity during pregnancy (Koopman et al., 2003; Rajagopalan and Long, 2012). These immunomodulatory receptors interact with foetal trophoblasts

invading the maternal uterine wall during placentation in a process that promotes the restructuring of the maternal circulatory system to provide the foetus with ample blood supply for growth and development (Parham, 2004).

The function of KIR pseudogenes is unknown, though these may provide genetic material for the evolution of other KIR genes via recombination events (Martin et al., 2003; Pyo et al., 2013). An additional KIR gene, KIR3DX1 (a.k.a KIR3DL0), is located between the Leukocyte Immunoglobulin-Like Receptor (LILR) clusters on Chromosome 19, towards the centromere in relation to the KIR gene cluster – the only identified KIR locus to exist outside of the KIR gene cluster (Sambrook et al., 2006).

The advent of next generation sequencing (NGS) now allows the Immunogenetics community to explore the true extent of KIR genetic polymorphism in closer detail than ever before (Roe et al., 2017; Wagner et al., 2018). However, application of this data in clinical research is stymied by the lack of KIR population dynamics data reference resources, such as detailed population frequency data for multiple ethnicities. There exists a wealth of reference material available for investigating HLA allelic frequencies and linkage disequilibrium, but comparatively little for KIR (Maiers et al., 2007; Gonzalez-Galarza et al., 2015; Hilton et al., 2015a). Data of this kind for KIR genes would aid advancement of a variety of clinical treatments, including cellular therapies, organ & cellular transplantation, and treatment of viral infection (Bari et al., 2013; Prakash et al., 2017; Boelen et al., 2018; Cooley et al., 2019). As the clinical implementation of KIR genetics develops, it is vital that the population dynamics of the KIR genetic system are well understood to ensure emerging research findings can be effectively translated into routine clinical utility.

This study aims to assess the KIR genetic frequencies observed by a single transplant centre to better understand the genetic variance observed within the centre's patient and donor population.

3.2 Methods

3.2.1 Cohort Selection

A total of 284 individuals were considered for KIR genotyping: 141 recipient and donor Haematopoietic Progenitor Cell Transplant (HPCT) pairs, and two healthy volunteers. Two HPCT recipients were excluded from the study as no pre-transplant genetic material was available for testing, and one further HPCT recipient was excluded from the study due to failed KIR genotyping as a result of poor-quality DNA (DNA denatured, confirmed by gel electrophoresis. Data not shown). Consequently, the final cohort size considered in the study was 281. All recipients in the HPCT pairs were patients (adult and paediatric) who received a transplant in treatment of Acute Myelogenous Leukaemia (AML) between 1st October 2011 and 31st March 2016 at the Manchester University NHS Foundation Trust (MFT). All donors were 9/10 or 10/10 HLA matched for the respective recipient. No donor in this cohort was selected on the basis of their KIR gene content. Ethnicity data of the cohort was not available.

3.2.2 KIR & HLA Genotyping

All samples were tested for both KIR gene presence/absence of the full KIR diplotype, and for KIR allelic definition at KIR2DL1, 2DL2, 2DL3, 2DL4, 3DL1S1, 3DL2, and 3DL3 to five-digit resolution. KIR presence/absence genotyping was carried out using the Luminex Sequence-Specific Oligonucleotide (PCR-SSO) LABType commercial kit [OneLambda, United States] and/or LinkSeq real time PCR (qPCR) [OneLambda, United States]. Local protocols were followed for PCR-SSO (modifying the

manufacturer's recommended protocol to use 40% of standard PCR-SSO bead volume), and manufacturer's recommended protocol followed for qPCR. Samples were tested by both PCR-SSO and qPCR when either equivocal results were identified on one test or to verify the results in cases where fewer than 3 samples were identified with the same respective KIR diplotype profile. In all other cases, successful genotyping by one technique was considered acceptable.

KIR allelic definition was carried out by amplicon-based short read (ABSR) NGS, using the KIR NGSgo®-AmpX commercial kit (GenDx), following manufacturer's recommended protocol. All KIR NGS genotyping was analysed using NGSengine v2.14 (GenDx), with the IPD-KIR 2.8.0 KIR database used as reference. All failed NGS tests were repeated at least once to attempt attainment of a successful result. A failed test was noted when the NGS testing failed twice, and the gene was positive for presence of the respective KIR locus. Failed tests were excluded from frequency analysis for the respective KIR locus.

Novel KIR alleles were confirmed by hybridisation-based targeted enrichment (HBTE) NGS, carried out by an external laboratory. This NGS testing was carried out at all loci for the respective sample, allowing verification of ABSR NGS results.

HLA class I genotyping data was collected to identify KIR ligands. Pre-existing HLA genotyping results were available for the entire cohort. All HLA genotyping data was collected from data of PCR-SSO LABType commercial kit (OneLambda) and/or sanger DNA sequencing commercial kits, using local protocols and acceptance criteria. HLA genotyping of the two volunteers was carried out using TruSight v2 commercial ABSR NGS kit (Illumina), using manufacturer's recommended protocols and acceptance criteria. Analysis of the HLA ABSR NGS was analysed using Assign

TruSight v2.1.0.943, and reference sequences from the 3.37 IMGT/HLA database release.

The HLA class I results were used to interpret the presence/absence of the C1, C2, Bw4 and A3/11 KIR epitopes, based upon 3.37 IMGT/HLA database data sets (Robinson et al., 2020). Bw4 definition considered both HLA-A and B locus sequences of each individual.

3.2.3 Statistical Analysis

Frequencies of KIR diplotypes and alleles, and KIR eptiopes, were calculated according to proportional representation of each respective characteristic with the sample size. When calculating allele carrier frequencies, the sample size was defined as the respective cohort size minus the number of failed tests. The statistical significance of the association between KIR alleles and KIR diplotypes was calculated by Fisher exact test, using MedCalc v19.5. Statistical significance was considered to be $p \leq 0.05$.

3.2.4 Definitions

KIR diplotype assignments (and in kind, KIR A/A & B/x diplotype stratification) were made in alignment with the Allele Frequency Net Database nomenclature (Gonzalez-Galarza et al., 2015). A/A diplotypes were defined by the absence of KIR2DL2,

2DL5, 3DS1, 2DS1, 2DS2, 2DS3, and 2DS5, with all other diplotype combinations defined as B/x diplotypes. KIR alleles were defined as described by the World Health Organisation (WHO) KIR nomenclature system (Marsh et al., 2003). Where ambiguities in allele definition exist, the primary assignment was made based on fewest mismatches in non-coding regions with the sequence available.

For the purposes of analysis, KIR gene sets for each individual were referred to as diplotypes. The testing used was inadequate for assigning genes or alleles to distinct haplotypes, and so definition of genotypes was also not possible.

3.2.5 Ethics

Ethical approval was obtained from the NHS Health Research Authority and Health and Care Research Wales (https://www.hra.nhs.uk/, application number: REC: 18/NW/0553, Appendix B.iii). The study was authorised by the Manchester University NHS Foundation Trust Clinical Trials Management Offices. Informed consent was obtained from all volunteers prior to donation of blood samples for gene testing and analysis. Informed consent for use of residual genetic material in research relating to transplantation was granted by all transplant recipients and donation at the point of consenting to the transplant.

3.3 Results

3.3.1 Testing

The study cohort demographic data can be found in Table 13. KIR diplotype data was available for 281 individuals. The KIR ABSR NGS genotyping presented a failure rate of 2.17% (41 failures across 1886 KIR loci, where the respective KIR gene was shown to be present by presence/absence genotyping). HBTE NGS for verification of potential novel

Characteristic	N (Percentage) (Total n=281)
Sex	
Female	102 (36.3%)
Male	176 (62.6%)
Cord Unit (Sex not recorded)	3 (1.1%)
Characteristic	
Transplant Recipient	138 (49.1%)
Transplant Donor	141 (50.2%)
Related Donor	36 (12.8%)
HLA Matched Sibling	34 (12.1%)
Other Relative	2 (0.7%)
Unrelated Donor	103 (36.7%)
HLA Matched Unrelated Donor	98 (34.9%)
Umbilical Cord Blood Unit	5 (1.8%)
Non-Transplant Volunteer	2 (0.7%)

Table 13 – Demographic information of study cohort.

alleles facilitated allelic definition of four failed loci, resulting in a final failure rate of 1.96% (37 failed KIR loci). Samples that were shown to be positive for a KIR gene by presence/absence genotyping, but failed NGS testing were excluded from further analysis of allelic definition for the respective failed gene(s) only.

KIR Diplotype ID	Centromeric							Centromeric/Telomeric			Telomeric						n	Freq	Accumulative Freq.
	KIR3DL3	KIR2DS2	KIR2DL2	KIR2DL3	KIR2DP1	KIR2DL1	KIR3DP1	KIR2DL5	KIR2DS3	KIR2DS5	KIR2DL4	KIR3DL1	KIR3DS1	KIR2DS1	KIR2DS4	KIR3DL2			
A/A1	P	a	a	P	P	P	P	a	a	a	P	P	a	a	P	P	90	0.320	0.320
B/x4	P	P	P	P	P	P	P	a	a	a	P	P	a	a	P	P	51	0.181	0.501
B/x2	P	a	a	P	P	P	P	P	a	P	P	P	P	P	P	P	37	0.132	0.633
B/x5	P	P	P	P	P	P	P	P	P	a	P	P	a	a	P	P	25	0.089	0.722
B/x3	P	P	P	P	P	P	P	P	a	P	P	P	P	P	P	P	13	0.046	0.768
B/x6	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	12	0.043	0.811
B/x7	P	P	P	P	P	P	P	P	P	a	P	P	P	P	P	P	9	0.032	0.843
B/x72	P	P	P	a	a	a	P	a	a	a	P	P	a	a	P	P	8	0.028	0.871
B/x28	P	a	a	P	P	P	P	P	P	P	P	P	P	P	P	P	4	0.014	0.885
B/x71	P	P	P	a	P	P	P	P	P	a	P	P	a	a	P	P	4	0.014	0.899
B/x70	P	P	P	P	P	P	P	P	P	P	P	a	P	P	a	P	3	0.011	0.910
B/x73	P	P	P	a	P	P	P	P	P	P	P	P	P	P	P	P	3	0.011	0.921
B/x90	P	P	P	a	P	P	P	P	P	a	P	P	P	P	P	P	3	0.011	0.932
B/x9	P	P	P	P	P	P	P	P	a	P	P	P	a	P	P	P	2	0.007	0.939
B/x14	P	a	a	P	P	P	P	a	a	a	P	P	P	a	P	P	2	0.007	0.946
B/x46	P	a	P	P	P	P	P	P	a	P	P	P	a	P	P	P	2	0.007	0.953
B/x56	P	P	a	P	P	P	P	P	P	P	P	P	P	P	P	P	2	0.007	0.960
B/x69	P	a	a	P	P	P	P	P	a	P	P	a	P	P	a	P	2	0.007	0.967
B/x81	P	P	P	a	P	P	P	P	P	P	P	a	P	P	a	P	2	0.007	0.974
B/x94	P	P	P	a	P	P	P	P	P	a	P	P	P	a	P	P	2	0.007	0.981
B/x8	P	a	a	P	P	P	P	P	P	a	P	P	P	P	P	P	1	0.004	0.985
B/x10	P	P	a	P	P	P	P	a	a	a	P	P	a	a	P	P	1	0.004	0.989
B/x13	P	P	P	P	P	P	P	P	P	a	P	P	P	a	P	P	1	0.004	0.993
B/x75	P	a	a	P	P	P	P	P	P	P	P	a	P	P	a	P	1	0.004	0.997
B/x76	P	P	P	a	a	a	P	P	a	P	P	P	P	P	P	P	1	0.004	1.000

Figure 12 – Observed KIR diplotype frequencies in the cohort population (n=281), including KIR gene content, listed in order of frequency rank. Haplotypes are reported in line with WHO nomenclature (Marsh et al., 2003). A total of twenty-five unique KIR diplotypes were identified, with the ten most common KIR diplotype profiles observed representing 89.9% (n=253) of the cohort. ***Key:*** *P=Gene present, a=Gene absent; Red=Inhibitory gene, Green=Activating gene, Purple=Pseudogene, Grey=Immunomodulatory gene during placentation of pregnancy.*

Of the 21 samples that failed NGS testing for at least one KIR locus, 95% of samples failed for one or two genes (13 and 6 samples respectively). Of the remaining

samples, one sample failed NGS at four KIR genes, and one sample failed for all six genes it was positive for. Both these samples included failed KIR3DL1 and 3DS1 tests), and were part of the same HPCT pair. There was a noticeable loci imbalance in the tests that failed, with approximately 38% (14) of the failed tests atKIR2DL1, 27% (10) typing at KIR3DL2, and 14% (5) typing at KIR3DS1.

3.3.2 Diplotype Diversity

A wide degree of diversity was observed, with twenty-five different KIR diplotypes identified: one A/A diplotype and twenty-four B/x diplotypes (Figure 12). The split between A/A and B/x diplotypes was 32.0% and 68.0% respectively. An imbalance in the frequency of diplotypes in the population was apparent, with the two most common diplotypes, A/A1 and B/x4, representing approximately half of the cohort (50.1%), and the three most common diplotypes (the afore-described diplotypes with the addition of B/x2) representing 63.3% of the population. Overall, the ten most common diplotypes represented 89.9% of the cohort population.

Gene	Positive no. *(%)*	Negative no. *(%)*
KIR2DL1	272 *(95.1%)*	9 *(4.9%)*
KIR2DL2	141 *(50.2%)*	140 *(49.8%)*
KIR2DL3	258 *(91.8%)*	23 *(8.2%)*
KIR2DL4	281 *(100%)*	0 *(0%)*
KIR2DL5	129 *(45.9%)*	152 *(54.1%)*
KIR2DS1	97 *(34.5%)*	184 *(65.6%)*
KIR2DS2	139 *(49.5%)*	142 *(50.5%)*
KIR2DS3	489 *(26.0%)*	208 *(74.0%)*
KIR2DS4	173 *(95.6%)*	8 *(4.4%)*
KIR2DS5	83 *(29.5%)*	198 *(70.5%)*
KIR3DL1S1	281 *(100%)*	0 *(0%)*
KIR3DL1	173 *(95.6%)*	8 *(4.4%)*
KIR3DS1	99 *(35.2%)*	182 *(64.8%)*
KIR3DL2	281 *(100%)*	0 *(0%)*
KIR3DL3	281 *(100%)*	0 *(0%)*
KIR2DP1	173 *(95.6%)*	8 *(4.4%)*
KIR3DP1	281 *(100%)*	0 *(0%)*

Table 14 – KIR gene presence/absence data of study cohort (n=281).
Framework genes are highlighted green. Non-framework genes found in the A haplotypes are highlighted yellow.

Table 14 presents the proportional presence/absence data for each KIR gene/pseudogene. All samples possessed all four framework genes (KIR2DL4, 3DL2, 3DL3, and 3DP1). The remaining genes of the KIR A haplotype were represented in

over 90% of the population (91.8-95.6%). With the exception of KIR2DL2 (found in 50.2% of samples), each gene associated with the KIR B haplotype were possessed by a minority of the cohort (26.0-49.5%).

3.3.3 Allelic Diversity

Allelic frequencies of the total cohort can be observed in Figure 13 to Figure 18. The degree of allelic diversity varied dramatically across different KIR loci, with KIR2DL2 demonstrating the most limited degree of polymorphism with three alleles identified (the third allele only defined in two siblings), and KIR3DL3 representing the most polymorphic gene with sixty-one alleles. The one activating KIR 'gene' analysed, KIR3DS1 (an allele group of KIR3DL1S1 that is often treated as an independent gene), presented limited polymorphism, with three alleles identified within the cohort, with a vast majority possessing KIR3DS1*01301 (93.6% of individuals possessing KIR3DS1 possessed this allele). The second most common allele, KIR3DS1*049N was identified in 8.5% of KIR3DS1 positive individuals, with one individual possessing both KIR3DS1*01301 and 049N. The third allele of KIR3DS1 was a novel allele identified in a single individual (described in **Table 15**).

For most KIR genes investigated, the majority of the cohort was represented in the most common two to five alleles, with the remaining alleles displaying nominal carrier frequencies (Figure 13 to Figure 18). The exceptions to this observation were KIR3DL2 and 3DL3, with an incremental decrease in frequency with each allele when ranked in order of carrier frequency.

A majority of the KIR allele designations provided limited number or no ambiguities. In contrast, extensive ambiguities were present for KIR3DL3 sequences, limiting the value of primary designations presented in this study.

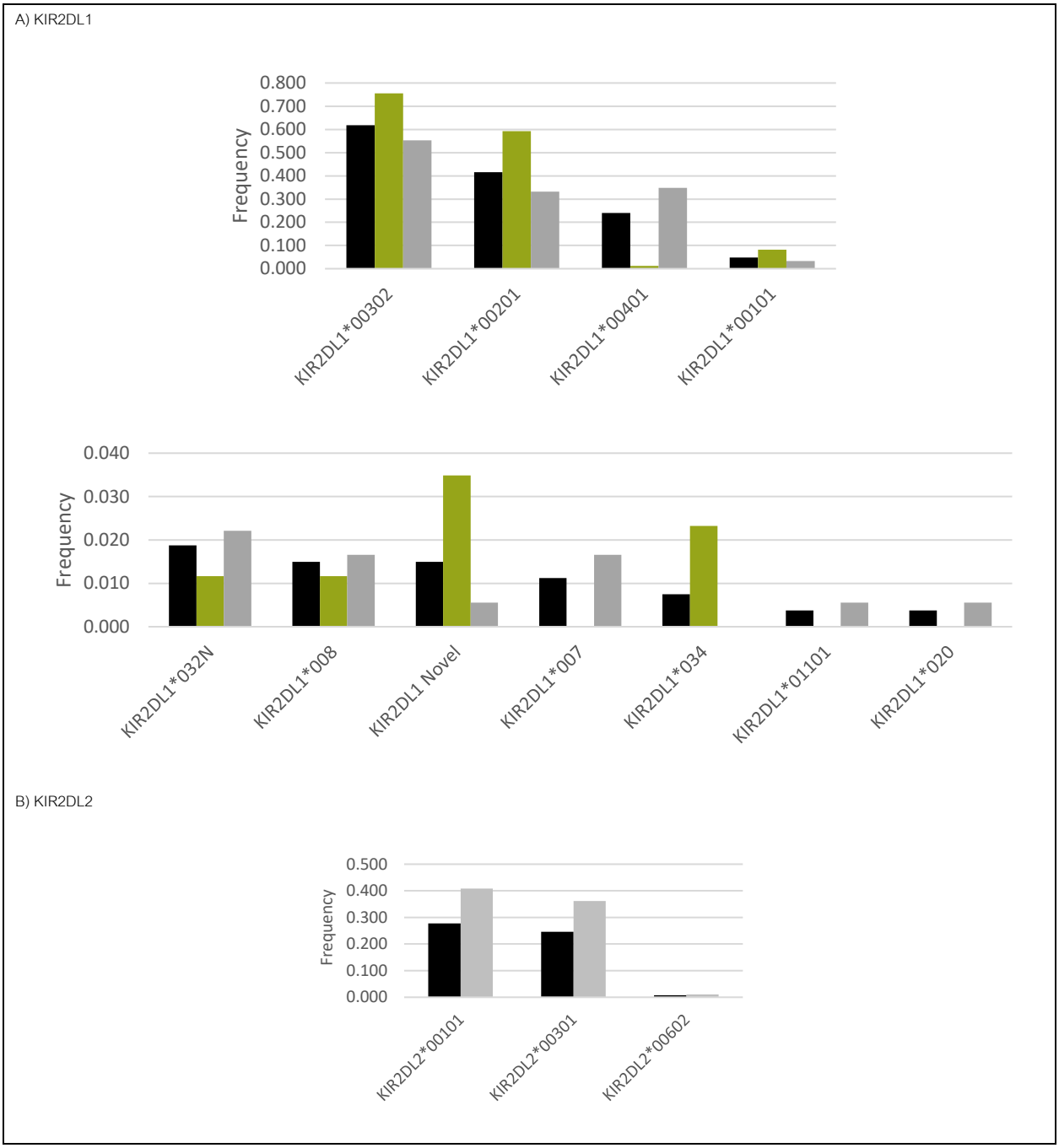


Figure 13 – Allelic frequency data of KIR2DL1 (A), and 2DL2 (B), comparing frequencies between the total cohort population (Black), those with KIR A/A diplotypes (Green), and those with KIR B/x diplotypes (Grey) (KIR2DL1: total pop. n=267, KIR A/A n=86, KIR B/x n=181. KIR2DL2: Total pop. n=281, B/x =191).

Samples positive for the relevant gene that failed NGS genotyping were excluded from analysis (14 samples for KIR2DL1, no samples for KIR2DL2). All KIR A/A diplotypes individuals were negative for KIR2DL2 by definition.

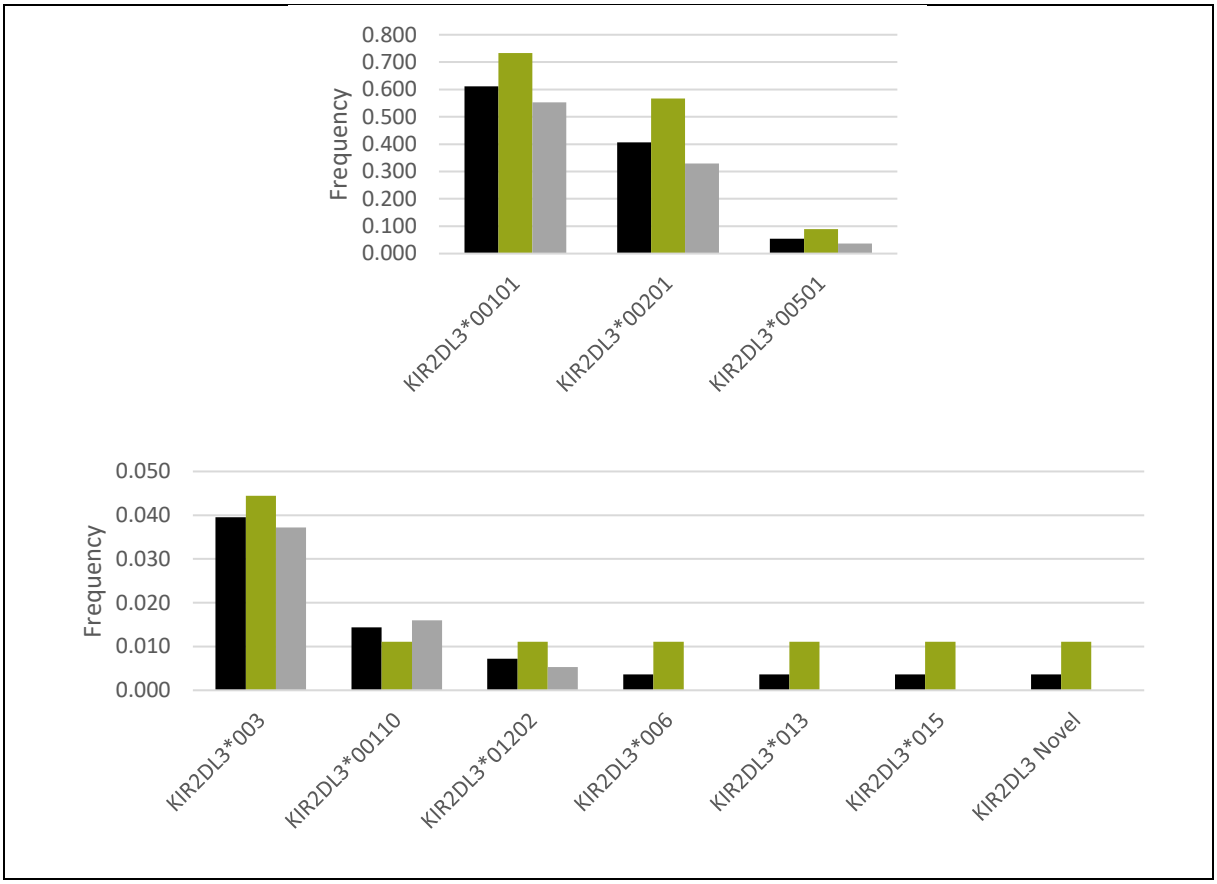


Figure 14 – Allelic frequency data of KIR2DL3, comparing frequencies between the total cohort population (Black, n=278), those with KIR A/A diplotypes (Green, n=90) and those with KIR B/x diplotypes (Grey, n=188).

Samples positive for KIR2DL3 that failed NGS genotyping were excluded from analysis (3 samples).

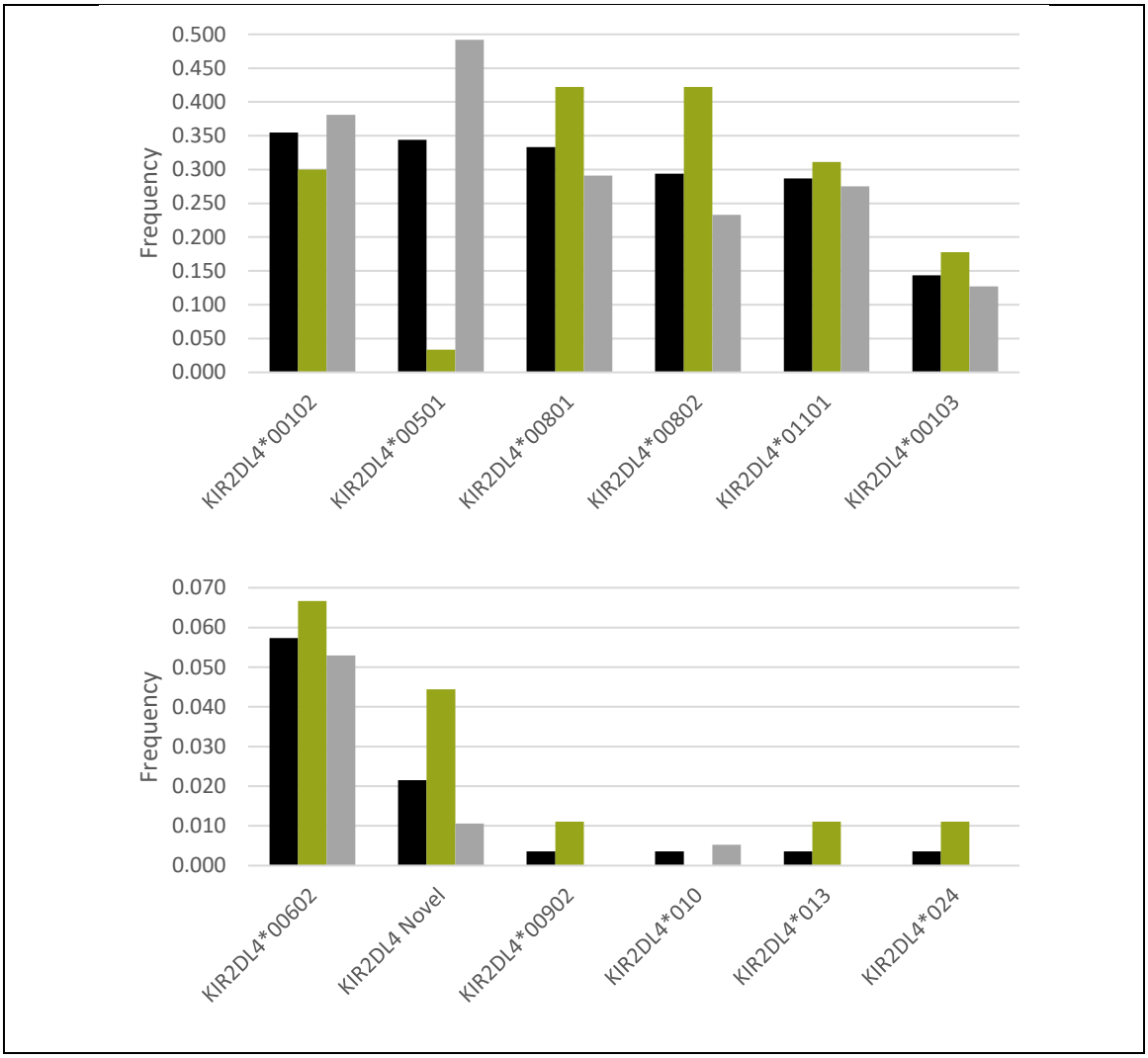


Figure 15 – Allelic frequency data of KIR2DL4, comparing frequencies between the total cohort population (Black, n=279), those with KIR A/A diplotypes (Green, n=90) and those with KIR B/x diplotypes (Grey, n=189).

Samples positive for KIR2DL4 that failed NGS genotyping were excluded from analysis (1 sample).

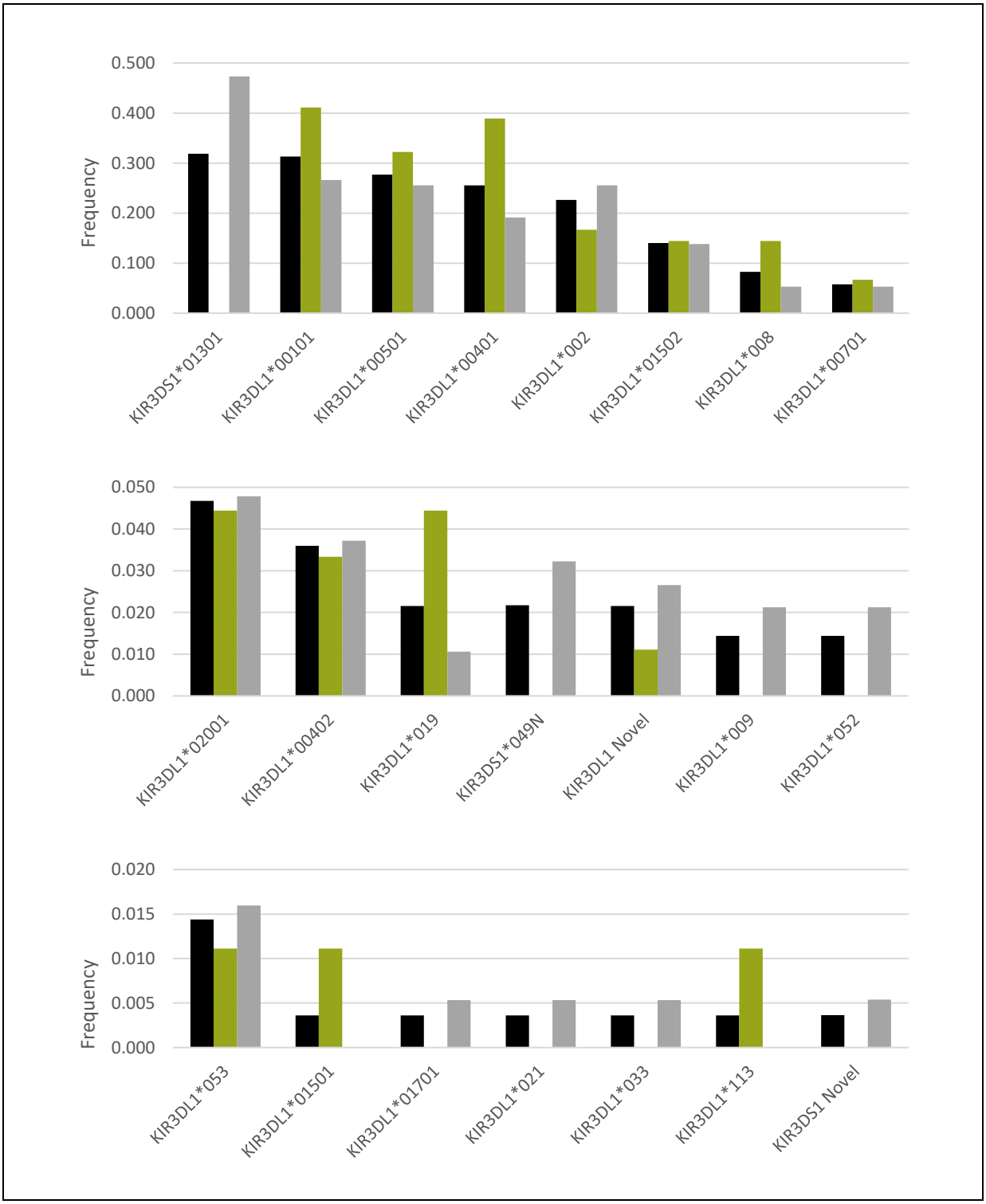


Figure 16 – Allelic frequency data of KIR3DL1S1, comparing frequencies between the total cohort population (Black), those with KIR A/A diplotypes (Green) and those with KIR B/x diplotypes (Grey) (KIR3DL1: total pop. n=278, KIR A/A n=90, KIR B/x n=188. KIR3DS1: Total pop. n=276, KIR A/A n=90, KIR B/x n=186).

Samples positive for the relevant gene that failed NGS genotyping were excluded from analysis (3 and 5 samples for KIR3DL1 and 3DS1, respectively).

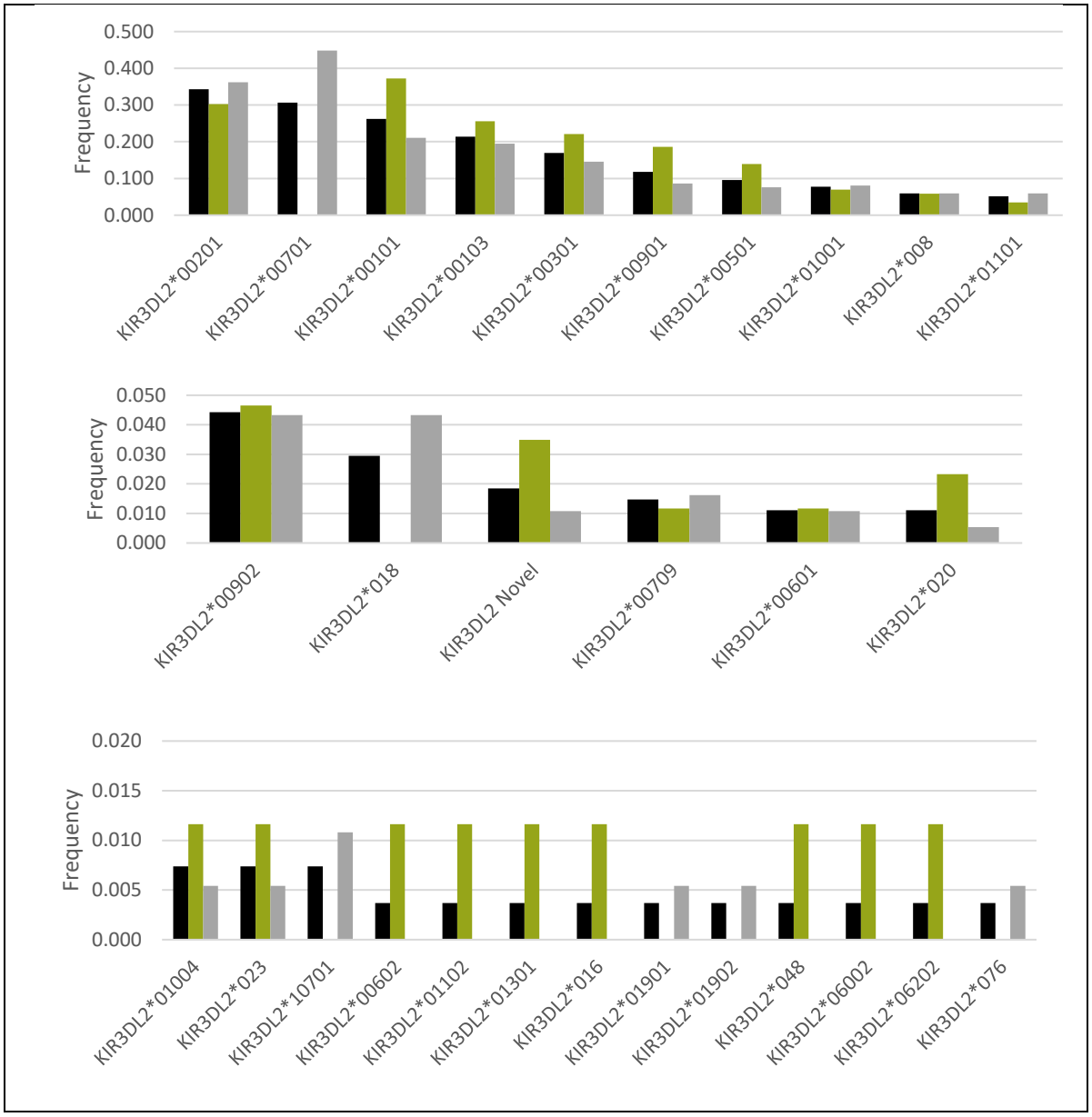


Figure 17 – Allelic frequency data of KIR3DL2, comparing frequencies of total cohort (Black, n=271), KIR A/A diplotype (Green, n=86), and KIR B/x diplotype (Grey, n=185) populations.

Samples positive for KIR3DL2 that failed NGS genotyping were excluded from analysis (11 samples).

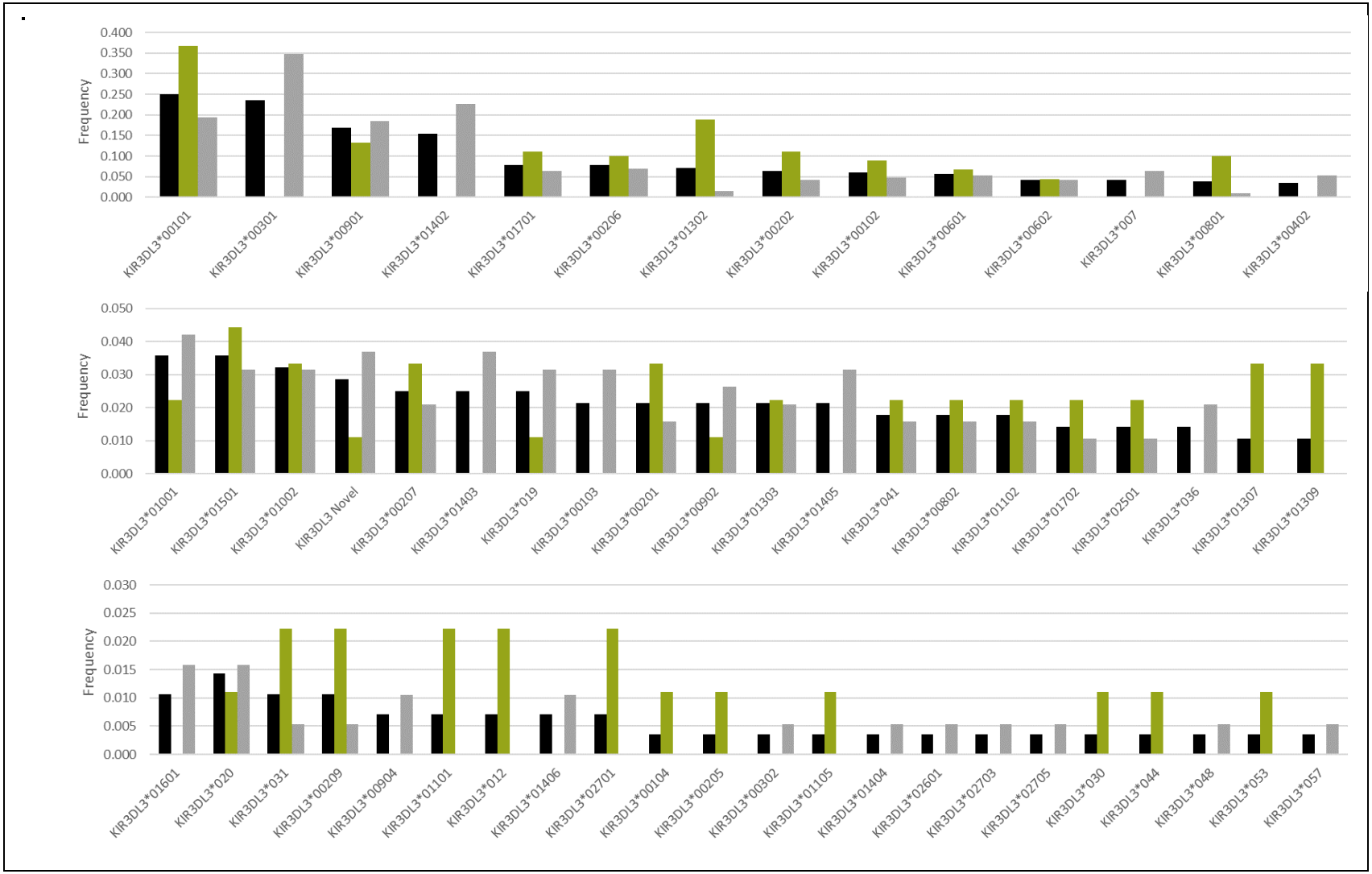


Figure 18 – Allelic frequency data of KIR3DL3, comparing frequencies of total cohort (Black, n=280), KIR A/A diplotype (Green, n=90), and KIR B/x diplotype (Grey, n=190) populations.

Samples that failed NGS genotyping at KIR3DL3 were excluded from analysis (1 sample).

Novel alleles were observed in all KIR genes investigated, except KIR2DL2, with twenty-five novel alleles identified in total (Table 15). Novel alleles with synonymous and non-synonymous mutations were identified (five and twenty, respectively). In all identified cases, these mutations were single nucleotide substitutions. In four instances, novel alleles were identified in more than one individual. One novel KIR3DL1 allele was identified in unrelated individuals, with the three other instances presented in paired siblings.

Identified novel alleles were subject to verification typing by HBTE NGS at all KIR loci, where residual genetic material was available. Verification testing was completed on 30 samples, representing twenty-five unique potential novel alleles. The HBTE NGS verified the initial ABSR NGS result in a majority of cases (Table 15). In two samples (one KIR2DL4 allele, and one KIR2DL2 allele), potential novel alleles were found to be due to a misinterpretation of Copy Number Variation (CNV) presenting three distinct alleles present in the genotype. In one case, the potential novel allele was identified to be due to the presence of a published allele which was not called by the ABSR analysis software. In all instances of disparity with justified cause, the result provided by HBTE NGS was used in the analysis presented by this study.

The HBTE NGS testing identified one sample possessing a recombinant KIR gene, KIR3DL1*060, a hybrid gene of KIR3DL1 and 3DL2. This was not identifiable by ABSR NGS, and so the true frequency of this allele within the cohort is unknown without further testing.

Gene Locus	Most similar published allele	Mutation in context of most similar previously defined alleles		Number of individuals in cohort possessing respective novel allele	Verified by HBTE NGS?
		Mutation Genomic Location Exon *(Domain)* cDNA position	**Phenotypical Outcome of Mutation** Codon position: Codon mutation *(Wild Type > Mutation)*		
KIR2DL1	00302	5 *(D2)* pos515	pos151: C**C**T > C**G**T *(Pro>Arg)*	1	Yes
	016	7 *(Transmembrane)* pos725	pos221: C**G**A > C**C**A *(Arg>Pro)*	1	Yes
	00201	8 *(Cytoplasmic)* pos824+825	pos254: G**CG >** G**TT** *(Ala>Val)*	1	Not Tested
	00302	9 *(Cytoplasmic)* pos910	pos283: **C**AG > **G**AG *(Gln>Glu)*	1	Yes
KIR2DL3	00101	6 *(Stem)* pos709	pos216: **G**AA > **A**AA *(Glu>Lys)*	1	Not Tested
KIR2DL4	00801	3 *(D0)* pos274	pos69: **G**CA > **A**CA *(Ala>Thr)*	2 (Siblings)	Yes
	00102	5 *(D2)* pos101	pos101: **G**AG > **A**AG *(Glu>Lys)*	1	Yes
	00802	5 *(D2)* pos651	pos194: GT**C** > GT**G** *(Val - Synonymous)*	1	Not Tested
	00501	9 *(Cytoplasmic)* pos1023	pos318: TT**G** > TT**A** *(Leu - Synonymous)*	1	Yes
KIR3DL1S1	01502 (KIR3DL1)	3 *(D0)* pos154	pos31: CGT > TGT *(Gly>Cys)*	1	Yes
	008 (KIR3DL1)	3 *(D0)* pos219	pos52: GG**C** > GG**T** *(Gly - Synonymous)*	1	Not Tested
	008 (KIR3DL1)	9 *(Cytoplasmic)* pos1242	pos373: **C**AG > **G**AG *(Gln>Glu)*	1	Yes
	002 (KIR3DL1)	9 *(Cytoplasmic)* pos1195	pos378: **G**CA > **C**CA *(Ala>Pro)*	3 (2 siblings & 1 unrelated)	Yes
	083 (KIR3DS1)	4 *(D1)* pos461	pos118: A**C**A > A**G**A *(Thr>Arg)*	1	Yes
KIR3DL2	00901	1 *(Leader)* pos16	pos-16: **G**TC > **A**TC *(Val>Ile)*	1	Yes
	00101	1 *(Leader)* pos27	pos-13: GC**G > GCA** *(Ala - Synonymous)*	1	Yes
	00103	4 *(D1)* pos388+407	pos109+115: **C**CA+T**C**A > **A**CA+T**A**A *(Pro+Ser > Thr+STOP - Truncation)*	1	Yes
	00301	5 *(D2)* pos704	pos214: C**A**G > C**T**G *(Gln>Leu)*	1	Yes
	10701	6 *(Stem)* pos962	pos300: A**G**T > AAT *(Ser>Asn)*	2 (Siblings)	Yes
	00101	9 *(Cytoplasmic)* pos1160	pos366: G**A**C > GGC *(Asp>Gly)*	1	Yes
KIR3DL3	01402	4 *(D1)* pos460	pos133: **C**TT > **A**TT *(Leu>Ile)*	2 (Siblings)	Yes
	00301	5 *(D2)* pos762	pos233: CA**T** > CA**C** *(His – Synonymous)*	1	Yes
	00103	5 *(D2)* pos908	pos282: G**C**G > G**T**G *(Ala>Val)*	1	Yes
	028	9 *(Cytoplasmic)* .pos1144	pos361: **G**CA > **A**CA *(Ala>Thr)*	**1**	Yes
	00206	9 *(Cytoplasmic)* pos1195	pos378: **C**AG > **G**AG *(Gln>Glu)*	**1**	Yes

Table 15 – Novel KIR genes identified in the cohort, including the theoretical phenotypical characteristic of the identified alleles.

Novel alleles demonstrating synonymous mutations are coloured grey.

An additional sample was identified to have an ambiguity at a single locus, KIR2DL1. ABSR NGS reported KIR2DL1*00201 homozygous, and HBTE NGS reported KIR2DL1*00201, 00302 (comparison listed in Table 16). This locus was tested twice by ABSR NGS: both times provided the same result, in spite of the first test not meeting acceptable pass criteria (minimum read depth of 23). This ambiguity presents potential haplotype drop out at this locus in ABSR NGS. The HBTE NGS heterozygous result was accepted, pending further testing.

ABSR NGS Result	HBTE NGS Result	Implicated Exon/Codon/ cDNA Position	ABSR NGS nucleotide balance at relevant position *no. read (%)*	HBTE NGS nucleotide balance at relevant position *no. read (%)*
KIR2DL1* 00201 hom.	KIR2DL1* 00201, 00302	Exon 3 Codon 16 pos110	C – 1508 (>99%) G – 2 (<1%)	C – 291 (54%) G – 249 (46%)
		Exon 3 Codon 27 pos144	A – 1565 (>99%) G – 3 (<1%)	A – 271 (49%) G – 280 (51%)
		Exon 4 Codon 114 pos404	C – 1361 (>99%) T – 12 (<1%)	C – 201 (44%) T – 261 (56%)

Table 16 – Identified ambiguities between amplicon-based short read (ABSR) NGS and hybridisation-based targeted enrichment (HBTE) NGS.

The magnitude of polymorphism observed in inhibitory KIR (iKIR) genes present within the KIR A/A and B/x diplotypes were broadly similar, though a number of alleles appeared to display clear association with KIR A/A diplotypes (fifteen alleles in total, across six genes) (Figure 13 to Figure 18, Table 17, Table 18). In comparison, only seven alleles presented significant association with B/x diplotypes, with four of these observed in KIR3DL3.

Alleles of KIR2DL2 were only observed in KIR B/x diplotypes, by definition of a KIR B haplotype.

	Identified in A/A Diplotypes only	Identified within both A/A & B/x Diplotypes	Identified in B/x Diplotypes only
KIR2DL1	034§	00101; 00201; 00302; 00401; 008§; 032N§	007§; 01101§; 020§
KIR2DL2	-	-	00101; 00301; 00602§
KIR2DL3	006§; 013§; 015§	00101/10§; 00201; 003; 00501; 01202§	-
KIR2DL4	00902§; 013§; 024§	00102/03; 00501; 00602; 00801/02; 01101	010
KIR3DL1S1	KIR3DL1*01501§; 113§	KIR3DL1*00101; 002; 00401/02; 00501; 00701; 008; 01502; 019§; 02001; 053§	KIR3DL1*009§; 01701§; 021§; 033§; 052§ KIR3DS1*01301; 049N§
KIR3DL2	00602§; 01102§; 01301§; 016§; 048§; 06002§; 06202§	00101/03; 00201; 00301; 00501; 00601§; 00701/09§; 008; 00901/02; 01001/04§; 01101; 020§; 023§	018§; 01901§/02§; 076§; 10701§
KIR3DL3	00104§; 00205§; 01101§/05§; 012§; 01307§; 02701§; 030§; 044§; 053§	00101/02; 00201/02/06/07§; 00601/02; 00801/02§; 00901/02; 01001/02§; 01102§; 01302/03/09§; 01402/03§/04§/05§/06§; 01501; 01701/02§; 019§; 02501§; 031§; 041§	00103§; 00209§; 00301/02§; 00402; 007; 00904§; 01601§; 020§; 02601§; 02703§/05§; 036§; 048§; 057§

Table 17 – Table categorising KIR alleles according to KIR haplotype grouping within the study cohort (n=281).

Key: § KIR allele defined in fewer than 10 individuals (3.6%) of the study cohort

Alleles displaying association with KIR A/A Diplotypes			
Allele	A/A Carrier Freq. (sample size)	B/x Carrier Freq. (sample size)	Significance
KIR2DL1*00201	0.593 *(n=86)*	0.331 *(n=181)*	p<0.001
KIR2DL1*00302	0.756 *(n=86)*	0.552 *(n=181)*	P=0.002
KIR2DL3*00101	0.733 *(n=90)*	0.553 *(n=188)*	p=0.004
KIR2DL3*00201	0.567 *(n=90)*	0.330 *(n=188)*	p<0.001
KIR2DL4*00801	0.422 *(n=90)*	0.291 *(n=190)*	p=0.041
KIR2DL4*00802	0.422 *(n=90)*	0.233 *(n=190)*	p=0.002
KIR3DL1*00101	0.411 *(n=90)*	0.266 *(n=188)*	p<0.001
KIR3DL1*00401	0.389 *(n=90)*	0.191 *(n=188)*	p<0.001
KIR3DL1*008	0.144 *(n=90)*	0.053 *(n=188)*	p=0.018
KIR3DL2*00101	0.372 *(n=86)*	0.211 *(n=185)*	P=0.007
KIR3DL2*00901	0.186 *(n=86)*	0.086 *(n=185)*	P=0.025
KIR3DL3*00101	0.367 *(n=90)*	0.195 *(n=190)*	p=0.005
KIR3DL3*00202	0.111 *(n=90)*	0.042 *(n=190)*	p=0.004
KIR3DL3*00801	0.100 *(n=90)*	0.011 *(n=190)*	p<0.001
KIR3DL3*01302	0.189 *(n=90)*	0.016 *(n=190)*	p<0.001

Alleles displaying association with KIR B/x Diplotypes			
Allele	A/A Carrier Freq. (sample size)	B/x Carrier Freq. (sample size)	Significance
KIR2DL1*00401	0.012 *(n=86)*	0.348 *(n=181)*	p<0.001
KIR2DL4*00501	0.033 *(n=90)*	0.492 *(n=189)*	p<0.001
KIR3DL2*00701	0.000 *(n=85)*	0.443 *(n=185)*	p<0.001
KIR3DL3*00301	0.000 *(n=90)*	0.347 *(n=190)*	p<0.001
KIR3DL3*00402	0.000 *(n=90)*	0.053 *(n=190)*	p=0.034
KIR3DL3*007	0.000 *(n=90)*	0.063 *(n=190)*	p=0.021
KIR3DL3*01402	0.000 *(n=90)*	0.226 *(n=190)*	p<0.001

Table 18 – KIR alleles displaying significant linkage disequilibrium with KIR A/A (left) or KIR B/x (right) diplotypes.

P values were calculated by Fisher Exact test.

3.3.4 KIR Ligand Diversity

Of the primary four inhibitory KIR epitopes (C1, C2, Bw4, and A3/11 epitopes of HLA class I), 76.9% of individuals in the cohort possessed two or three iKIR epitopes (Figure 19). Only 12.8% of the cohort possessed one iKIR epitope, and 10.3% possess all four epitopes. Of the HLA-C epitopes, a majority of individuals were C1/C1 homozygous (42.3%) or C1/C2 heterozygous (43.8%). C2/C2 homozygous individuals represented just 13.9% of the cohort. Closer inspection of HLA-Bw4 polymorphism demonstrates a relatively even distribution of HLA-Bw4 epitopes with threonine or isoleucine at position 80 (47.0% and 34.5% of the cohort, respectively) (Figure 20).

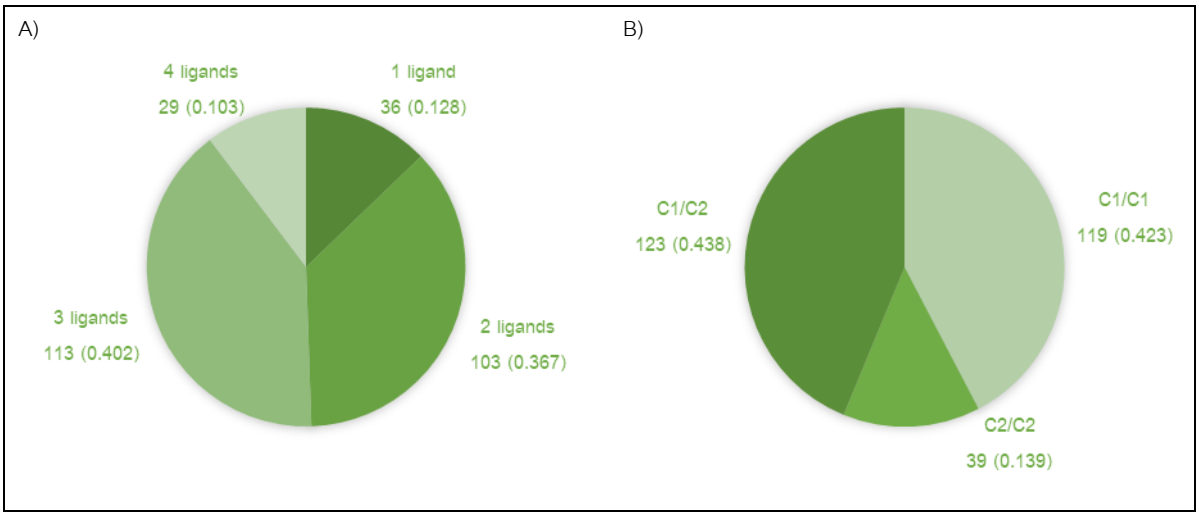


Figure 19 – KIR ligand frequencies of the cohort (n=281).

A) Proportion of cohort possessing 1-4 KIR epitopes (C1, C2, Bw4, and A3/11). B) Proportion of C1 and C2 epitope in the cohort.

Figure 21 displays the frequencies of KIR/ligand combinations for the cohort. In all four iKIR assessed, most individuals possessed both the receptor and cognate ligand (55.5-85.8%). Frequencies of receptor/ligand combinations is largely dictated by the frequency of the KIR ligand, with few individuals negative for the receptor. As such, the iKIR gene product with the largest proportion of receptor positive/ligand negative

was KIR3DL2 and A3/11 ligand due to the relative scarcity of HLA-A3 and A11 compared to other ligands.

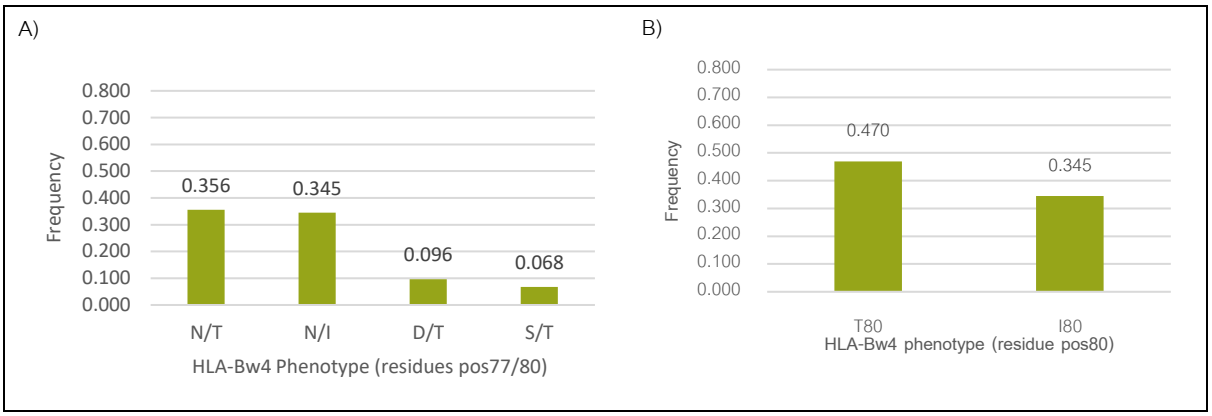


Figure 20 – Frequencies of HLA-Bw4 phenotypes in the study cohort (n=281).

Frequencies presented as HLA-Bw4 characterised by residues 77 and 80 (A), and by position 80 alone (B). Evaluation of HLA-Bw4 considered the structure of both HLA-A and HLA-B.

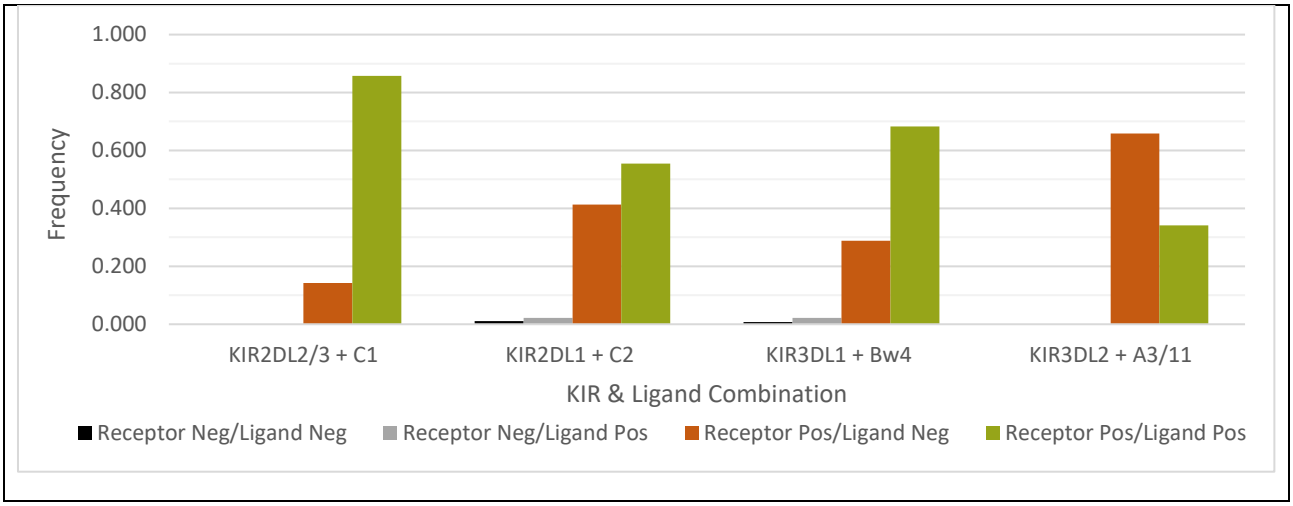


Figure 21 – Frequencies of KIR and KIR ligand presence/absence combinations (n=281).

The presence of the KIR ligand is vital for the licensing of NK cells presenting the respective KIR glycoprotein (Elliott and Yokoyama, 2011).

3.3.5 Comparison of carrier frequencies with other European populations

Comparison of KIR allelic carrier frequencies compared to those of studies concerning other European populations can be found in Table 19 to Table 21. This suggests the observed genomic data is likely to present an approximate facsimile of the populations experienced by other clinical H&I laboratories throughout the UK, and of the British population at large.

	KIR Diplotypes									
Population (n)	A/A1	B/x4	B/x2	B/x5	B/x3	B/x6	B/x7	B/x72	B/x28	B/x71
Study Cohort (n=281)	0.320	0.181	0.132	0.089	0.046	0.043	0.032	0.028	0.014	0.014
U.K. (n=90, 99, 136, 154, 186, 200, 334, 584) (Guinan et al., 2010; Gonzalez-Galarza et al., 2015)	0.274 – 0.372	0.077 – 0.165	0.082 – 0.152	0.052 – 0.111	0.025 – 0.120	0.030 – 0.065	0.010 – 0.074	0.010 – 0.024	0.005 – 0.013	0.015 – 0.040
Republic of Ireland (n=136) (Guinan et al., 2010)	0.316	0.059	0.059	0.044	0.132	0.044	0.022	N/R	0.006	N/R
Germany (n=99, 120) (Uhrberg et al., 2002; Gonzalez-Galarza et al., 2015)	0.313-0.317	0.152-0.169	0.004 - 0.063	0.075	0.022	0.020 – 0.045	0.030	0.019	N/R	0.049
France (n=102, 108, 130) (Gonzalez-Galarza et al., 2015)	0.200 – 0.320	0.112 – 0.137	0.072 – 0.137	0.064 – 0.098	0.026 – 0.088	0.039 – 0.079	0.048 – 0.060	0.010 – 0.016	0.08 – 0.026	0.016 – 0.020
Spain (n=100, 126, 230, 278, 339, 414) (Santin et al., 2006; Gonzalez-Galarza et al., 2015; Cisneros et al., 2020)	0.242 – 0.313	0.048 – 0.173	0.071 – 0.174	0.083 – 0.230	0.038 – 0.167	0.008 – 0.080	0.008 – 0.099	0.004 – 0.060	0.004 – 0.007	0.007 – 0.038
Croatia (n=111, 125, 121) (Burek et al., 2013; Gonzalez-Galarza et al., 2015)	0.234 – 0.336	0.128 – 0.153	0.045 – 0.080	0.081 – 0.149	0.048 – 0.081	0.048 – 0.054	0.045 – 0.066	0.016 – 0.027	N/R	0.016 – 0.018

Table 19 – Comparison of observed KIR diplotypes frequencies observed in the cohort population versus other reported European populations.

Key: N/R = Not Reported

	KIR Diplotypes									
Population (n)	B/x70	B/x73	B/x90	B/x9	B/x14	B/X46	B/x56	B/x69	B/x81	B/x94
Study Cohort (n=281)	0.011	0.011	0.011	0.007	0.007	0.007	0.007	0.007	0.007	0.007
U.K. (n=90, 99, 136, 154, 186, 200, 334, 584) (Guinan et al., 2010; Gonzalez-Galarza et al., 2015)	0.010 – 0.017	0.010 – 0.020	0.007 – 0.033	0.005 – 0.030	0.005 – 0.006	N/R	0.010	0.011 – 0.020	0.005 – 0.009	0.007 – 0.010
Republic of Ireland (n=136) (Guinan et al., 2010)	N/R	N/R	N/R	0.007	0.022	N/R	N/R	N/R	N/R	N/R
Germany (n=99, 120) (Uhrberg et al., 2002; Gonzalez-Galarza et al., 2015)	0.004 – 0.010	0.022	0.008	0.004 – 0.008	N/R	N/R	N/R	N/R	0.004	N/R
France (n=102, 108, 130) (Gonzalez-Galarza et al., 2015)	0.029	0.010 – 0.024	0.024 – 0.029	0.010 – 0.032	0.032 – 0.060	N/R	N/R	N/R	0.008 – 0.026	0.010
Spain (n=100, 126, 230, 278, 339, 414) (Santin et al., 2006; Gonzalez-Galarza et al., 2015; Cisneros et al., 2020)	0.007 – 0.022	0.002 – 0.030	0.007 – 0.026	0.004 – 0.087	0.016 – 0.042	N/R	N/R	0.012 – 0.030	0.007 – 0.022	0.002 – 0.014
Croatia (n=111, 121, 125) (Burek et al., 2013; Gonzalez-Galarza et al., 2015)	0.008	0.018	0.009	N/R	0.008 – 0.009	N/R	N/R	0.009	N/R	N/R

Table 20 – Comparison of observed KIR diplotypes frequencies observed in the cohort population versus other reported European populations.

Key: N/R = Not Reported

	KIR Diplotypes					
	B/x8	B/x10	B/x13	B/x75	B/x76	
Population (n)						
Study Cohort (n=281)	0.004	0.004	0.004	0.004	0.004	
U.K. (n=90, 99, 136, 154, 186, 200, 334, 584) (Guinan et al., 2010; Gonzalez-Galarza et al., 2015)	0.005 – 0.027	0.002 – 0.030	0.005 – 0.022	0.005 – 0.015	0.007 – 0.010	
Republic of Ireland (n=136) (Guinan et al., 2010)	0.037	0.022	0.007	N/R	N/R	
Germany (n=99, 120) (Uhrberg et al., 2002; Gonzalez-Galarza et al., 2015)	N/R	N/R	N/R	N/R	0.004	
France (n=102, 108, 130) (Gonzalez-Galarza et al., 2015)	0.019	N/R	0.024	N/R	N/R	
Spain (n=100, 126, 230, 278, 339, 414) (Santin et al., 2006; Gonzalez-Galarza et al., 2015; Cisneros et al., 2020)	0.004 – 0.030	0.004 – 0.010	0.002 – 0.008	0.010 – 0.003	0.005 – 0.012	
Croatia (n=111, 121, 125) (Burek et al., 2013; Gonzalez-Galarza et al., 2015)	0.016 – 0.018	N/R	0.008 – 0.018	N/R	0.008	

Table 21 – Comparison of observed KIR diplotypes frequencies observed in the cohort population versus other reported European populations.

Key: N/R = Not Reported

3.4 Discussion

Our study has demonstrated the highly polymorphic nature of the KIR gene complex, with a high degree of gene content variation and allelic diversity observed. The single transplant centre cohort presented population frequency data that is comparable to other reported European populations. Investigating the genetic diversity of KIR is a crucial step in the understanding of the interactions between KIR genetics and phenotypical characteristics, and in the construction of more intricate clinical algorithms for patient care (such as donor selection algorithms in HPCT).

Both variation in the gene content of the KIR gene cluster and allelic polymorphisms within each KIR gene contribute to the genetic diversity of KIR, influencing the

phenotypical characteristics of the lymphocytes which express these genes. Licensing of NK cells via interactions between the KIR glycoprotein and their cognate ligands, HLA class I, further increase the complexities of this gene family's effect upon NK cell cytotoxic activity (Nowak et al., 2014; Nowak et al., 2015; Boudreau and Hsu, 2018).

3.4.1 Diplotype Diversity

32.0% of the cohort presented with a KIR A/A diplotype, with the remaining 68.0% possessing a variant of the KIR B/x diplotypes. There was substantial imbalance between the frequencies of different diplotypes, with the four most common KIR diplotype profiles (A/A, B/x4, B/x2, and B/x5) representing 72.2% of the cohort, despite there being twenty-five observed diplotypes.

The relatively small cohort size limits the number of KIR diplotypes and alleles observed, but there is a marked decline in the observed frequencies after the 10 most common KIR diplotypes. These ten diplotypes represent 89.9% of the total cohort, indicating these most common diplotypes likely represent the vast majority of the overall population. The remaining fifteen diplotypes appear to repeat the centromeric and telomeric gene content of other more common diplotypes. Unfortunately, the available typing data was insufficient to assign KIR2DL5, 2DS1 and 2DS5 loci to centromeric and telomeric regions for further diplotype characterisation.

3.4.2 Allelic Diversity

The allelic polymorphism presents allele ranking similar to other published data examining large cohorts of European populations, with reported frequencies closely aligning with those reported by other UK population studies (Table 19 to Table 21) (Uhrberg et al., 2002; Santin et al., 2006; Guinan et al., 2010; Burek et al., 2013;

Gonzalez-Galarza et al., 2015; Wagner et al., 2018). This indicates the reported study's data may be indicative of the wider UK population, but would benefit from a larger cohort to more closely correlate with the frequencies of the wider population and identify any significant variance with the data of other European populations. Alternatively, UK population frequencies may differ from those observed in other European nations, but this postulation cannot be proven without a larger cohort.

Similar to the KIR diplotype frequency data, the most common one to five alleles of each KIR gene represent a large majority of each gene within the cohort. This suggests a limited subset of KIR alleles may represent the vast majority of the UK population, in spite of KIR's highly polymorphic characteristics.

A limited number of allelic ambiguities were present for most KIR genes, reducing the risk of incorrect allelic definition. In contrast, a wide degree of polymorphism observed in KIR3DL3, combined with the difficulty in definitive phasing of cis/trans heterozygous positions separated by large intervals, result in a large number of ambiguities as part of the allele assignment, which may interfere with the reported frequency data. As such, the frequencies reported are only suggestive of true allelic frequencies in the cohort. The use of long read third generation sequencing (TGS) would be required to definitively define alleles. More extensive frequency reference data would aid imputation of allele assignment in short read NGS techniques to facilitate identification of the most common alleles where ambiguities are present. Similar tools using HLA frequency data exist, designating the common and well documented alleles of each HLA locus, providing a valuable utility to clinical H&I laboratories (Mack et al., 2013). Nevertheless, ABSR NGS is sufficient for

investigating SNP motifs where cis/trans ambiguities (and thus allele assignments) may be irrelevant to requirements.

A large number of novel alleles were identified relative to the small cohort size, with twenty-five unique novel alleles identified in a cohort of 281 individuals, with a verified by HBTE NGS. This demonstrates the limited reporting and publishing of KIR sequence data internationally. For a unified and standardised approach to KIR genetic nomenclature, and to fully understand the polymorphic nature of KIR genetic evolution. The novel KIR alleles identified in our study will be reported to IPD-KIR database following completion of the doctorate thesis.

A number of recombinant KIR genes (a.k.a. fusion or hybrid genes) have been identified, arising from intragenic recombination resulting in a gene consisting of portions from two distinct genes (Norman et al., 2009; Pyo et al., 2013; Cisneros et al., 2020). This may significantly change the protein transcribed from this gene, for example combining the binding domains of an activating KIR gene with the intracellular signalling domains of an inhibitory gene (Cisneros et al., 2020). In the thirty samples sequenced by HBTE NGS, one such recombinant gene was identified in a single individual, KIR3DL1*060 (originally named KIR3DL1/2 *v*), a hybrid gene that encodes a glycoprotein with the binding domains of KIR3DL1 and the stem, transmembrane region and intracellular domains of KIR3DL2 (Artavanis-Tsakonas et al., 2003). This allele was not defined by ABSR NGS. The use of ABSR NGS lacks specificity for defining recombinant genes, as recombinant data is likely to be mis-interpreted by sequencing analysis software during phasing imputation. As such, the scarcity of any recombinant genes reported in this study does not preclude the presence of recombinant genes within the study cohort. Further sequencing by TGS or HBTE

NGS would be required to identify these genes, preferably long-range amplicon PCR third generation sequencing to sequence the full gene cluster in a single read.

3.4.3 KIR Ligand Diversity

Possessing both the KIR gene and the cognate ligand is vital for the licensing of NK cells to reach their full functional capacity (Boudreau and Hsu, 2018; Goodson-Gregg et al., 2020). The majority of the cohort possessed the investigated inhibitory KIR glycoproteins for NK cell licensing, with all individuals positive for KIR3DL2, and 96.8% and 97.2% of individuals positive for KIR2DL1 and KIR3DL1 respectively. All individuals were positive for a C2-specific KIR (KIR2DL2 and/or KIR2DL3). Consequently, possessing the respective KIR ligand was the primary predictor of possessing both the receptor and ligand necessary for licensing (Figure 21).

A majority (76.9%) of the cohort possessed 2-3 KIR ligands of the four investigated (HLA class 1 containing C2, C1, Bw4, or A3/11 epitopes), demonstrating similar frequencies to other studies (Closa et al., 2020). When examining C epitopes alone, 43.8% were C1/C2 heterozygous, 42.3% were C1/C1 homozygous, and 13.9% were C2/C2 homozygous. Due to the relative low frequency of HLA bearing the C2 and A3/11 epitopes, KIR2DL1 and KIR3DL2 presented the lowest receptor positive/ligand positive (RP/LP) frequencies at 55.5% and 34.2% of individuals respectively. The Bw4 (68.3% RP/LP) and C1 (85.8% RP/LP) epitopes are more common. Other studies have identified similar KIR and ligand compatibility frequencies in European populations (Closa et al., 2020).

As a further complication of NK cell licensing theory, HLA-Bw4 itself is a polymorphic epitope (Figure 20) (Gumperz et al., 1995; Gumperz et al., 1997). N--TALR and N--IALR (pos77-83) were the most common form of Bw4 observed in

the cohort with frequencies of approximately 35% each, with less common Bw4 epitopes, D--TLLR and S--IALR each found in less than 10% of the cohort. When stratified by position 80 alone, the frequencies of HLA-Bw4-T^{80} (43.8%) and HLA-Bw4-I^{80} (39.5%) were observed in similar proportions. It is still not fully understood what impact this ligand polymorphism has on the interactions with KIR3DL1, but initial studies suggest different forms of the Bw4 epitope demonstrate variance in both their binding affinity with the KIR glycoprotein and surface density of the relevant HLA bound to the target cell (Boudreau et al., 2016).

3.4.4 Further Work

Our study provides an indication of genetic frequencies encountered by a single UK transplant centre. At 281 individuals, including a relatively large proportion of related individuals (25.6% of the cohort representing part of related pairs), these results provide only an indication of the overall diversity of KIR genes throughout the population of the United Kingdom. Additionally, many of the of the donors in this study were from international registries, which is likely to impact the overall genetic frequencies of the cohort, possibly beyond that which would be observed in the UK. With further work, the scope of the cohort could be expanded to consider a larger cohort of the local population.

The nature of the gene-specific PCR amplification used in this study's KIR genotyping methodology make it difficult to discern haplotypes and genotypes from the genomic data collected. Previous studies have identified several haplotype designations that can arise from the same KIR diplotype profile, and so it is difficult to impute haplotype and genotype information from the available data with sufficient sensitivity (Roe et al., 2017). Understanding the true extent of haplotype variation may be of a limited

utility in clinical algorithms but would provide information for researching the genetic evolution and population genetics of the KIR gene cluster.

For full analysis of the population data, it is vital that this data expands upon the extent of CNV within the KIR gene cluster. The CNV analysis carried out as part of HBTE NGS, using sequence amplification imbalance as an indicator of copy number variation, highlighted that many of the heterozygous KIR loci possessed two copies of one allele and one copy of the other (data not shown). CNV analysis was not possible as part the ABSR NGS analysis, resulting in a greater risk of incorrect calls due to amplification imbalance of alleles. To account for this imbalance, a relatively low base variation threshold of >20% was used in analysis to define a heterozygous position. Analysis software can conflate sequences, especially when more than two alleles are present. In two cases, sequences that were initially interpreted as a potential novel allele at a heterozygous locus (with two alleles) by ABSR NGS were found to be due to the presence of three published alleles by HBTE NGS.

One KIR locus discrepancy was identified by gene sequencing with ABSR NGS and verification HBTE NGS: providing a KIR2DL1*00201 homozygous definition by ABSR NGS, and a KIR2DL1*00201, 00302 heterozygous definition by HBTE NGS. ABSR NGS is potentially more prone to allele imbalance leading to incorrect homozygous designation due to preferential amplification during the initial amplification PCR (Muyas et al., 2019). Genotyping by a third technique is required to formally confirm the correct allele designation.

The verification of potentially novel alleles identified within this cohort improves the integrity of this data set in cases where an anomaly was noted, but it is a risk that other loci with three alleles may have been missed in ABSR NGS due to amplification

imbalance. Development of the analysis software used in ABSR NGS to consider the presence of >2 alleles in a single sample would be required to reduce the risk of erroneous calls.

3.4.5 Limitations

Multiple potential sources of bias are present in the cohort selection process. Approximately half (49.1%) of the cohort were HPCT recipients in the treatment for AML, so it is possible that this cohort may present a bias towards KIR alleles that predispose a susceptibility to developing leukaemia. Data correlating KIR genomics with onset of cancer and oncogenetics is conflicting (Augusto, 2016).

The remaining half of the cohort (excluding two volunteers) were donors selected on the basis of their HLA match with the respective recipient. Despite the MHC and KIR gene complexes residing on separate chromosomes, there is evidence that HLA and KIR have likely co-evolved (Augusto and Petzl-Erler, 2015; Hilton et al., 2015a). The study cohort may have presented skewed frequencies due to this interaction (Single et al., 2007; Hilton et al., 2015a). This potentially limits the scope of the cohort's genetic diversity compared to that observed in the general population.

Furthermore, 12.8% of the cohort were donors related to their respective recipient. Mendelian inheritance dictates that even an HLA matched sibling (12.1% of the cohort) only has a 25% likelihood of being all matched for KIR genotype. This roughly correlates with the observed cohort, with twelve sibling pairs confirmed to possess full KIR matches (35.3% of all sibling pairs in the study).

The inclusion of HPCT donors is highly likely to present bias in the frequencies of KIR ligands observed, due to their selection for donation on the basis of HLA match with the recipient. Of the 141 donors included in their study, only 5 presented

mismatches at HLA-B and 13 at HLA-C. The majority of donors therefore possessed identical sets of KIR ligands as their respective recipient. While the population data of HLA ligands remains of valuable, as shown by the similar frequencies observed in other studies, the cohort size for these measurements has effectively been halved by this bias.

Despite the multiple biases described, the similarity in our dataset with those of other European population studies is an indication that any potential bias is likely to have a minimal impact on the overall results (Table 19 to Table 21).

3.4.6 Conclusion

The KIR gene cluster presents a large degree of variation, both in gene content and allelic polymorphism. Presenting data from even a relatively small cohort is extremely valuable to the clinical H&I field, providing an indication of frequencies in the wider population that can be used as a foundation to further research and development of clinical algorithms. It is hoped that this study data can be used alongside other population studies to aid other groups in these developments.

Chapter 3 References

Artavanis-Tsakonas, K., Eleme, K., McQueen, K. L., Cheng, N. W., Parham, P., Davis, D. M. & Riley, E. M. (2003) Activation of a subset of human NK cells upon contact with Plasmodium falciparum-infected erythrocytes. *J Immunol,* 171(10), 5396-405.

Augusto, D. G. (2016) The Impact of KIR Polymorphism on the Risk of Developing Cancer: Not as Strong as Imagined? *Front Genet,* 7, 121.

Augusto, D. G. & Petzl-Erler, M. L. (2015) KIR and HLA under pressure: evidences of coevolution across worldwide populations. *Hum Genet,* 134(9), 929-40.

Barbarin, A., Cayssials, E., Jacomet, F., Nunez, N. G., Basbous, S., Lefevre, L., Abdallah, M., Piccirilli, N., Morin, B., Lavoue, V., Catros, V., Piaggio, E., Herbelin, A. & Gombert, J. M. (2017) Phenotype of NK-Like CD8(+) T Cells with Innate Features in Humans and Their Relevance in Cancer Diseases. *Front Immunol,* 8, 316.

Bari, R., Rujkijyanont, P., Sullivan, E., Kang, G., Turner, V., Gan, K. & Leung, W. (2013) Effect of donor KIR2DL1 allelic polymorphism on the outcome of pediatric allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol,* 31(30), 3782-90.

Bjorkstrom, N. K., Beziat, V., Cichocki, F., Liu, L. L., Levine, J., Larsson, S., Koup, R. A., Anderson, S. K., Ljunggren, H. G. & Malmberg, K. J. (2012) CD8 T cells express randomly selected KIRs with distinct specificities compared with NK cells. *Blood,* 120(17), 3455-65.

Boelen, L., Debebe, B., Silveira, M., Salam, A., Makinde, J., Roberts, C. H., Wang, E. C. Y., Frater, J., Gilmour, J., Twigger, K., Ladell, K., Miners, K. L., Jayaraman, J., Traherne, J. A., Price, D. A., Qi, Y., Martin, M. P., Macallan, D. C., Thio, C. L., Astemborski, J., Kirk, G., Donfield, S. M., Buchbinder, S., Khakoo, S. I., Goedert, J. J., Trowsdale, J., Carrington, M., Kollnberger, S. & Asquith, B. (2018) Inhibitory killer cell immunoglobulin-like receptors strengthen CD8(+) T cell-mediated control of HIV-1, HCV, and HTLV-1. *Sci Immunol,* 3(29), eaao2892.

Boudreau, J. E. & Hsu, K. C. (2018) Natural Killer Cell Education and the Response to Infection and Cancer Therapy: Stay Tuned. *Trends Immunol,* 39(3), 222-239.

Boudreau, J. E., Mulrooney, T. J., Le Luduec, J. B., Barker, E. & Hsu, K. C. (2016) KIR3DL1 and HLA-B density and binding calibrate NK education and response to HIV. *J Immunol,* 196(8), 3398-410.

Burek, M. K., Grubic, Z., Stingl, K. & Zunec, R. (2013) Distribution of KIR genes in the Croatian population. *Hum Immunol,* 74(8), 952-6.

Campbell, K. S. & Hasegawa, J. (2013) Natural killer cell biology: an update and future directions. *J Allergy Clin Immunol,* 132(3), 536-544.

Cisneros, E., Moraru, M., Gómez-Lozano, N., Muntasell, A., López-Botet, M. & Vilches, C. (2020) Haplotype-Based Analysis of KIR-Gene Profiles in a South European Population—Distribution of Standard and Variant Haplotypes, and Identification of Novel Recombinant Structures. *Frontiers in Immunology,* 11.

Closa, L., Vidal, F., Herrero, M. J. & Caro, J. L. (2020) Distribution of human killer cell immunoglobulin-like receptors and ligands among blood donors of Catalonia. *HLA,* 95(3), 179-188.

Cooley, S., He, F., Bachanova, V., Vercellotti, G. M., DeFor, T. E., Curtsinger, J. M., Robertson, P., Grzywacz, B., Conlon, K. C., Waldmann, T. A., McKenna, D. H., Blazar, B. R., Weisdorf, D. J. & Miller, J. S. (2019) First-in-human trial of rhIL-15 and haploidentical natural killer cell therapy for advanced acute myeloid leukemia. *Blood Adv,* 3(13), 1970-1980.

Elliott, J. M. & Yokoyama, W. M. (2011) Unifying concepts of MHC-dependent natural killer cell education. *Trends Immunol,* 32(8), 364-72.

Gonzalez-Galarza, F. F., Takeshita, L. Y., Santos, E. J., Kempson, F., Maia, M. H., da Silva, A. L., Teles e Silva, A. L., Ghattaoraya, G. S., Alfirevic, A., Jones, A. R. & Middleton, D. (2015) Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. *Nucleic Acids Res,* 43(Database issue), D784-8.

Goodson-Gregg, F. J., Krepel, S. A. & Anderson, S. K. (2020) Tuning of human NK cells by endogenous HLA-C expression. *Immunogenetics,* 72(4), 205-215.

Guinan, K. J., Cunningham, R. T., Meenagh, A., Dring, M. M., Middleton, D. & Gardiner, C. M. (2010) Receptor systems controlling natural killer cell function are genetically stratified in Europe. *Genes Immun,* 11(1), 67-78.

Gumperz, J. E., Barber, L. D., Valiante, N. M., Percival, L., Phillips, J. H., Lanier, L. L. & Parham, P. (1997) Conserved and variable residues within the Bw4 motif of HLA-B make separable contributions to recognition by the NKB1 killer cell-inhibitory receptor. *J Immunol,* 158(11), 5237-41.

Gumperz, J. E., Litwin, V., Phillips, J. H., Lanier, L. L. & Parham, P. (1995) The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med,* 181(3), 1133-44.

Hilton, H. G., Guethlein, L. A., Goyos, A., Nemat-Gorgani, N., Bushnell, D. A., Norman, P. J. & Parham, P. (2015a) Polymorphic HLA-C receptors balance the functional characteristics of KIR haplotypes. *J Immunol,* 195(7), 3160-70.

Huard, B. & Karlsson, L. (2000) KIR expression on self-reactive CD8+ T cells is controlled by T-cell receptor engagement. *Nature,* 403(6767), 325-8.

Ivarsson, M. A., Michaelsson, J. & Fauriat, C. (2014) Activating killer cell Ig-like receptors in health and disease. *Front Immunol,* 5, 184.

Koopman, L. A., Kopcow, H. D., Rybalov, B., Boyson, J. E., Orange, J. S., Schatz, F., Masch, R., Lockwood, C. J., Schachter, A. D., Park, P. J. & Strominger, J. L. (2003) Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med,* 198(8), 1201-12.

Krasnova, Y., Putz, E. M., Smyth, M. J. & Souza-Fonseca-Guimaraes, F. (2017) Bench to bedside: NK cells and control of metastasis. *Clin Immunol,* 177, 50-59.

Lisnic, B., Lisnic, V. J. & Jonjic, S. (2015) NK cell interplay with cytomegaloviruses. *Curr Opin Virol,* 15, 9-18.

Mack, S. J., Cano, P., Hollenbach, J. A., He, J., Hurley, C. K., Middleton, D., Moraes, M. E., Pereira, S. E., Kempenich, J. H., Reed, E. F., Setterholm, M., Smith, A. G., Tilanus, M. G., Torres, M., Varney, M. D., Voorter, C. E., Fischer, G. F., Fleischhauer, K., Goodridge, D., Klitz, W., Little, A. M., Maiers, M., Marsh, S. G., Muller, C. R., Noreen, H., Rozemuller, E. H., Sanchez-Mazas, A., Senitzer, D., Trachtenberg, E. & Fernandez-Vina, M. (2013) Common and well-documented HLA alleles: 2012 update to the CWD catalogue. *Tissue Antigens,* 81(4), 194-203.

Maiers, M., Gragert, L. & Klitz, W. (2007) High-resolution HLA alleles and haplotypes in the United States population. *Hum Immunol,* 68(9), 779-88.

Marsh, S. G., Parham, P., Dupont, B., Geraghty, D. E., Trowsdale, J., Middleton, D., Vilches, C., Carrington, M., Witt, C., Guethlein, L. A., Shilling, H., Garcia, C. A., Hsu, K. C. & Wain, H. (2003) Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Immunogenetics,* 55(4), 220-6.

Martin, M. P., Bashirova, A., Traherne, J., Trowsdale, J. & Carrington, M. (2003) Cutting edge: expansion of the KIR locus by unequal crossing over. *The Journal of Immunology,* 171(5), 2192-5.

Misra, M. K., Augusto, D. G., Martin, G. M., Nemat-Gorgani, N., Sauter, J., Hofmann, J. A., Traherne, J. A., Gonzalez-Quezada, B., Gorodezky, C., Bultitude, W. P., Marin, W., Vierra-Green, C., Anderson, K. M., Balas, A., Caro-Oleas, J. L., Cisneros, E., Colucci, F., Dandekar, R., Elfishawi, S. M., Fernandez-Vina, M. A., Fouda, M., Gonzalez-Fernandez, R., Grosse, A., Herrero-Mata, M. J., Hollenbach, S. Q., Marsh, S. G. E., Mentzer, A., Middleton, D., Moffett, A., Moreno-Hidalgo, M. A., Mossallam, G. I., Nakimuli, A., Oksenberg, J. R., Oppenheimer, S. J., Parham, P., Petzl-Erler, M. L., Planelles, D., Sanchez-Garcia, F., Sanchez-Gordo, F., Schmidt, A. H., Trowsdale, J., Vargas, L. B., Vicario, J. L., Vilches, C., Norman, P. J. & Hollenbach, J. A. (2018) Report from the killer-cell immunoglobulin-like receptors (KIR) component of the 17th International HLA and Immunogenetics Workshop. *Hum Immunol,* 79(12), 825-33.

Muyas, F., Bosio, M., Puig, A., Susak, H., Domenech, L., Escaramis, G., Zapata, L., Demidov, G., Estivill, X., Rabionet, R. & Ossowski, S. (2019) Allele balance bias identifies systematic genotyping errors and false disease associations. *Hum Mutat,* 40(1), 115-126.

Nash, W. T., Teoh, J., Wei, H., Gamache, A. & Brown, M. G. (2014) Know Thyself: NK-Cell Inhibitory Receptors Prompt Self-Tolerance, Education, and Viral Control. *Front Immunol,* 5, 175.

Norman, P. J., Abi-Rached, L., Gendzekhadze, K., Hammond, J. A., Moesta, A. K., Sharma, D., Graef, T., McQueen, K. L., Guethlein, L. A., Carrington, C. V., Chandanayingyong, D., Chang, Y. H., Crespi, C., Saruhan-Direskeneli, G., Hameed, K., Kamkamidze, G., Koram, K. A., Layrisse, Z., Matamoros, N., Mila, J., Park, M. H., Pitchappan, R. M., Ramdath, D. D., Shiau, M. Y., Stephens, H. A., Struik, S., Tyan, D., Verity, D. H., Vaughan, R. W., Davis, R. W., Fraser, P. A., Riley, E. M., Ronaghi, M. & Parham, P. (2009) Meiotic recombination generates rich diversity in NK cell receptor genes, alleles, and haplotypes. *Genome Res,* 19(5), 757-69.

Nowak, J., Koscinska, K., Mika-Witkowska, R., Rogatko-Koros, M., Mizia, S., Jaskula, E., Polak, M., Mordak-Domagala, M., Lange, J., Gronkowska, A., Jedrzejczak, W. W., Kyrcz-Krzemien, S., Markiewicz, M., Dzierzak-Mietla, M., Tomaszewska, A., Nasilowska-Adamska, B., Szczepinski, A., Halaburda, K., Hellmann, A., Czyz, A., Gil, L., Komarnicki, M., Wachowiak, J., Baranska, M., Kowalczyk, J., Drabko, K., Gozdzik, J., Wysoczanska, B., Bogunia-Kubik, K., Graczyk-Pol, E., Witkowska, A., Marosz-Rudnicka, A., Nestorowicz, K., Dziopa, J., Szlendak, U., Warzocha, K., Lange, A. & Polish Donor-Recipient Matching Study, G. (2015) Role of donor activating KIR-HLA ligand-mediated NK cell education status in control of malignancy in hematopoietic cell transplant recipients. *Biol Blood Marrow Transplant,* 21(5), 829-39.

Nowak, J., Koscinska, K., Mika-Witkowska, R., Rogatko-Koros, M., Mizia, S., Jaskula, E., Polak, M., Mordak-Domagala, M., Lange, J., Gronkowska, A., Jedrzejczak, W. W., Kyrcz-Krzemien, S., Markiewicz, M., Dzierzak-Mietla, M., Tomaszewska, A., Nasilowska-Adamska, B., Szczepinski, A., Halaburda, K., Hellmann, A., Komarnicki, M., Gil, L., Czyz, A., Wachowiak, J., Baranska, M., Kowalczyk, J., Drabko, K., Gozdzik, J., Wysoczanska, B., Bogunia-Kubik, K., Graczyk-Pol, E., Witkowska, A., Marosz-Rudnicka, A., Nestorowicz, K., Dziopa, J., Szlendak, U., Warzocha, K., Lange, A. A. & Polish Donor-Recipient Matching Study, G. (2014) Donor NK cell licensing in control of malignancy in hematopoietic stem cell transplant recipients. *Am J Hematol,* 89(10), E176-83.

Parham, P. (2004) NK cells and trophoblasts: partners in pregnancy. *J Exp Med,* 200(8), 951-5.

Prakash, S., Sarangi, A. N., Alam, S., Sonawane, A., Sharma, R. K. & Agrawal, S. (2017) Putative role of KIR3DL1/3DS1 alleles and HLA-Bw4 ligands with end stage renal disease and long term renal allograft survival. *Gene,* 637, 219-229.

Pyo, C. W., Wang, R., Vu, Q., Cereb, N., Yang, S. Y., Duh, F. M., Wolinsky, S., Martin, M. P., Carrington, M. & Geraghty, D. E. (2013) Recombinant structures expand and contract inter and intragenic diversification at the KIR locus. *BMC Genomics,* 14, 89.

Rajagopalan, S. & Long, E. O. (2012) KIR2DL4 (CD158d): An activation receptor for HLA-G. *Front Immunol,* 3, 258.

Robinson, J., Barker, D. J., Georgiou, X., Cooper, M. A., Flicek, P. & Marsh, S. G. E. (2020) IPD-IMGT/HLA Database. *Nucleic Acids Res,* 48(D1), D948-D955.

Roe, D., Vierra-Green, C., Pyo, C. W., Eng, K., Hall, R., Kuang, R., Spellman, S., Ranade, S., Geraghty, D. E. & Maiers, M. (2017) Revealing complete complex KIR haplotypes phased by long-read sequencing technology. *Genes Immun,* 18(3), 127-34.

Sambrook, J. G., Bashirova, A., Andersen, H., Piatak, M., Vernikos, G. S., Coggill, P., Lifson, J. D., Carrington, M. & Beck, S. (2006) Identification of the ancestral killer immunoglobulin-like receptor gene in primates. *BMC Genomics,* 7, 209.

Santin, I., de Nanclares, G. P., Calvo, B., Gaafar, A., Castano, L., Group, G.-N. & Bilbao, J. R. (2006) Killer cell immunoglobulin-like receptor (KIR) genes in the Basque population: association study of KIR gene contents with type 1 diabetes mellitus. *Hum Immunol,* 67(1-2), 118-24.

Single, R. M., Martin, M. P., Gao, X., Meyer, D., Yeager, M., Kidd, J. R., Kidd, K. K. & Carrington, M. (2007) Global diversity and evidence for coevolution of KIR and HLA. *Nat Genet,* 39(9), 1114-9.

Uhrberg, M., Parham, P. & Wernet, P. (2002) Definition of gene content for nine common group B haplotypes of the Caucasoid population: KIR haplotypes contain between seven and eleven KIR genes. *Immunogenetics,* 54(4), 221-229.

Wagner, I., Schefzyk, D., Pruschke, J., Schofl, G., Schone, B., Gruber, N., Lang, K., Hofmann, J., Gnahm, C., Heyn, B., Marin, W. M., Dandekar, R., Hollenbach, J. A., Schetelig, J., Pingel, J., Norman, P. J., Sauter, J., Schmidt, A. H. & Lange, V. (2018) Allele-level KIR genotyping of more than a million samples: workflow, algorithm, and observations. *Front Immunol,* 9(2843), 1-15.

Chapter 4 | Comparison of clinical algorithms assessing killer-cell immunoglobulin-like receptor genetics for donor selection in T cell depleted reduced intensity conditioning haematopoietic progenitor cell transplantation

Summary

A number of different killer-cell immunoglobulin-like receptors (KIR) assessment algorithms have been published for use in donor selection for haematopoietic progenitor cell transplantation (HPCT), but no model has been found to be universally successful in all centres. The diversity of models available underlies the restricted success of these models, with anecdotal and published reports of KIR assessment models not presenting the desired outcome in all transplant centres. Consequently, it is good practice to validate any proposed model using a local transplant population before implementation. This study directly compared four such models using a single centre cohort: donor KIR A/A diplotypes versus KIR B/x diplotypes, the receptor/ligand mismatching (RLM) model, and two variations of the 2010 B content scoring model. The primary cohort for the study were T cell depleted reduced intensity conditioning (RIC) transplants (n=88), with a secondary cohort including all transplants meeting the selection criteria (n=115) also examined for comparison. The use of donors with KIR B/x diplotypes in T cell depleted RIC HPCT was found to increase the risk of 3-year GvHD grade II-IV (53.2% versus 19.4% respectively, p=0.021, p_c=0.084; HR=3.22, 95% CI=1.13-9.17, p=0.029, p_c=0.145) and increase incidence of 3-year NRM (23.5% versus 0.00% respectively, p=0.032, p_c=0.128) compared to transplants using donors with KIR A/A diplotypes. Receptor/ligand mismatched transplants trended towards reducing incidence of 3-year NRM compared to receptor/ligand matched transplants (14.6% versus 30.2%, p=0.064, p_c=0.256; HR=0.40, 95% CI=0.13-1.19, p=0.098, p_c=0.490). No model investigated in the presented cohort demonstrated any influence upon overall survival, event-free survival, or relapse. Although a relatively small cohort of 88 transplant pairs was investigated, these findings corroborate the evidence presented by other studies and help to build a larger picture concerning the impact of KIR assessment models in HPCT.

4.1 Introduction

The clinical utility of natural killer (NK) cells in haematopoietic progenitor cell transplantation (HPCT) programmes has been considered since the late 1980s (Murphy et al., 1987; Murphy et al., 1990; Rolstad and Benestad, 1991; Murphy et al., 1992). The investigation into the influence of NK cells' killer-cell immunoglobulin-like (KIR) receptors and their epitopes gained popularity in the following decade, with the first proposed KIR epitope mismatching model (Ruggeri et al., 1999). In the following years, several competing models were proposed, including the KIR receptor/ligand mismatching (RLM) model and the KIR B content scoring model (Leung et al., 2004; Cooley et al., 2009; Cooley et al., 2010). This trend has persisted to the present, with the most recent novel KIR assessment model for donor selection, the IM-KIR Score, offered this year (Krieger et al., 2020).

The KIR gene cluster is composed of up to thirteen KIR genes and two pseudogenes. Many of the KIR genes contribute to the cytotoxic functional capacity of an NK cell through encoding of glycoprotein receptors that either inhibit or activate cytotoxic action upon interaction with their cognate ligand (Kärre et al., 1986; Ciccone et al., 1995; Moretta et al., 1995; Mandelboim et al., 1996). The gene content of the KIR gene complex varies widely both within and between ethnic populations (Gonzalez-Galarza et al., 2015; Vierra-Green et al., 2016; Gentle et al., 2017; Machado-Sulbaran et al., 2017). KIR haplotypes are broadly categorised as A or B: KIR A haplotypes are highly conserved in their gene content, presenting up to 7 genes and two pseudogenes: KIR2DL1, 2DL3, 2DL4, 2DS4, 3DL1, 3DL2, 3DL3, 2DP1 and 3DP1. KIR B haplotypes exhibit extensive diversity in gene content, identifiable by the addition of further activating KIR (aKIR) and/or inhibitory KIR (iKIR) genes:

KIR2DL2, 2DS1, 2DS2, 2DS3, and/or 2DS5 (Gonzalez-Galarza et al., 2015). The increased number of activating KIR genes found in KIR B haplotypes compared to KIR A haplotypes has led to the monikers of 'inhibitory' haplotypes for KIR A haplotypes and 'activating' haplotypes for KIR B haplotypes.

The iKIR glycoproteins interact with HLA class I, their cognate ligand, across the HLA's α1 and α2 helices (Boyington et al., 2000). The residue at position 80 of HLA-Cw represents a primary ligand for several iKIR, with approximately half of HLA-Cw antigens possessing asparagine (C1 epitope) at this position, and the remainder antigens possessing lysine (C2 epitope) (Mandelboim et al., 1996). Additional iKIR epitopes are found in position 77 and 80 of HLA-Bw4 public epitopes of HLA-B and some HLA-A antigens, and the A3/11 epitope found within HLA-A3 and 11 antigens (Hansasuta et al., 2004; Saunders et al., 2015).

Many KIR assessment algorithms are now available for HPCT donor selection, but a majority share the broad principle of promoting increased NK cell reactivity as a consequence of either: (i) reducing NK cell inhibition by reducing interaction of iKIR with their cognate ligands (for example, selecting donors with iKIR incompatibility for the recipient's HLA class I profile), or (ii) encouraging the activation of NK cells by selection of donors with an increased number of activating KIR (aKIR) genes, often defined in relation to KIR B haplotype characteristics (Leung et al., 2004; Cooley et al., 2009; Cooley et al., 2010; Cooley et al., 2014; Nowak et al., 2014; Nowak et al., 2015). Few transplant centres in the United Kingdom routinely implement these models as part of their HPCT programmes due to the financial outlay and increased workload involved in the requisite additional testing, with the reported clinical benefits deemed insufficient to justify this expenditure.

The extensive diversity of published KIR assessment algorithms is emblematic of the disjointed approach to KIR genomics within the clinical Histocompatibility and Immunogenetics (H&I) field. Though some models, such as the RLM model and B content scoring model, are more commonly implemented, it is recognised within the field that no KIR assessment algorithm is universally applicable. All models published thus far have been found to be effective in some centres and fail to be reproduce the proposed post-transplant benefits in other centres (Davies et al., 2020; Schetelig et al., 2020). This unsuccessful data is rarely published, making it difficult to ascertain a root cause for these disparities, or identify commonalities between successful/unsuccessful implementations. The investigation into the key determinants of successful KIR assessment implementation is obfuscated by a number of highly complex factors and interactions, including the vast heterogeneity in both HPCT treatment protocols, and the diseases they treat. Ultimately, the differing cohorts between transplant centres and studies make direct comparisons between published findings difficult to parse. Recent studies have identified interactions between the influence of KIR genetics upon post-transplant outcomes and wider clinical characteristics of the transplantation process, with contradictory findings identified with different clinical settings. For example, T cell depleted versus T cell replete transplantation, and RIC versus myeloablative conditioning (MAC), both severely alter the observations regarding KIR characteristics to the extent of providing contradictory results (Cooley et al., 2014; Bultitude et al., 2020).

In the interest of good clinical practice, each transplant centre should validate the clinical effectiveness of any donor selection algorithm they choose to implement, to demonstrate it is a justifiable pursuit for their local requirements. This study aims to directly compare several established KIR models for HPCT donor selection on a single

centre cohort in a retrospective analysis: KIR A/A versus KIR B/x donors, the RLM model, and two iterations of the B-content scoring model: the 2010 KIR B Scoring Model (assessing B content score ≥2 and weighting for CenB/B), and the 2010 KIR B scoring model with 'Better' and 'Best' categories combined into a single group (assessing B content score ≥2 without weighting for CenB/B) (Leung et al., 2004; Cooley et al., 2009; Cooley et al., 2010).

4.2 Methods

4.2.1 Cohort Selection

The criteria for inclusion in this study was as follows: (i) all HPCT carried out in the treatment of AML in adult patients (≥18 years) at the Manchester Royal Infirmary, (ii) for the recipient's first transplant, (iii) from a single cell source, (iv) using a donor that was a 9/10 or 10/10 HLA match with the recipient (genotyped to two field resolution), (v) transplanted between 1st October 2011 and 31st March 2016.

Double cord blood unit transplants (i.e. multiple cell sources) were excluded from this study due to the complexities of considering the co-existence and interactions of two donor grafts within a single recipient. Second/subsequent grafts were excluded for similar reasons. Re-transplantation was considered an end event, indicative of a failed first graft, with the respective transplant pair excluded from further follow-up and analysis at this time point. As HLA compatibility between donor and recipient is regarded as a major influence of post-transplant outcomes, transplants involving grafts inferior to 9/10 HLA match with the recipient were excluded to minimise the heterogeneity of the cohort.

133 transplants were carried out at the Manchester Royal Infirmary in the defined time period. Twelve transplants were excluded in total. Three double cord transplants were excluded, and a further five transplants were excluded due to being re-transplantation events. Four transplants were excluded for using a donor inferior to a 9/10 HLA match with the recipient: two 8/10 HLA matched single umbilical cord blood unit transplants, one 8/10 HLA-matched unrelated donor (MUD), and one haplo identical sibling transplant (8/10 HLA matched).

Of the 121 transplant pairs that met all described acceptance criteria, six transplant pairs were excluded due to a lack of donor material for relevant testing. This excluded 5% of the potential cohort. The final study cohort investigated was 115 transplant pairs.

To assess the interaction between transplant work-up protocol and the influences of KIR genomics, the complete cohort (Cohort A, n=115) was assessed in parallel with a sub-cohort of transplants with T cell depletion and reduced intensity conditioning (Cohort B, n=88).

Three-year post-transplant follow-up was carried out for the following post-transplant outcomes: GvHD grade II-IV, relapse and secondary malignancies, overall survival (OS) and event-free survival (EFS). All post-transplant outcome data was collected from recipient medical records and local departmental data.

4.2.2 KIR & HLA Genotyping

All samples had full KIR diplotype genotyping (gene presence/absence resolution). All testing for KIR genotyping was carried out on residual genetic material, with consent for transplant-related research provided at time of consent for transplantation. KIR presence/absence genotyping was carried out using the Luminex reverse

Sequence-Specific Oligonucleotide (PCR-SSO) LABType commercial kit (One Lambda) and/or LinkSeq real time PCR (qPCR) (Linkage Biosciences). Local protocols were followed for PCR-SSO. The qPCR procedure followed the manufacturer's recommend protocol. Samples were tested by both PCR-SSO and qPCR when either equivocal results were identified on one test or to verify the results in cases where fewer than 3 samples were identified with the respective KIR diplotype gene content profile. In all other cases, successful genotyping by one technique was considered acceptable. KIR diplotype identities were assigned as described by the Allele Frequency Net Database (Gonzalez-Galarza et al., 2015).

HLA class I genotyping data was collected to identify KIR ligands. Historic HLA genotyping results were available for the entire cohort. All HLA genotyping was carried out using PCR-SSO LABType commercial kit (OneLambda) and/or Sanger DNA sequencing commercial kits, using local validated protocols and acceptance criteria. The HLA class I results were then used to deduce the presence/absence of the C1, C2, Bw4 and A3/11 KIR ligands, based upon EBI reference sequence data sets (IMGT reference v3.37.0) (Robinson et al., 2020).

4.2.3 Statistical Analysis

Frequencies for each characteristic were calculated according to proportional representation of each respective type with the sample size. All Kaplan Meier survival curves and hazard ratios (HR) were calculated using IBM SPSS Statistics v25. Kaplan Meier survival curves were constructed for primary and secondary transplant outcomes factors, with log rank regression and cox regression (with associated hazard ratio data) also calculated. Statistical significance was considered to be $p \leq 0.05$. Holm-Bonferonni sequential correction was carried out for all statistics to provide a

corrected p value (p_c) (Gaetano, 2018). The p_c values are only presented where the respective $p<0.10$.

4.2.4 Definitions

Four different models for assessing KIR in HPCT were investigated: three assessing KIR B haplotype content, and one assessing donor's iKIR mismatching with the patient's iKIR ligands. The KIR A/A and B/x diplotype characteristics are assigned on the basis of the Allele Frequency Net Database KIR diplotype ID (Gonzalez-Galarza et al., 2015).

Two variations of the 2010 KIR B Content Scoring Model were investigated (Cooley et al., 2010). The first used the KIR B Content Scoring Model as described, stratifying based on donor KIR B content score >2 with weighting to the centromeric region of the diplotype to stratify donors into Neutral, Better, or Best, calculated using the online Donor KIR B-content group calculator (Cooley et al., 2010). The second variation combined the Better and Best categories into a single stratum: stratifying based on donor KIR B content score >2, with no weighting to the centromeric region of the KIR gene cluster.

The KIR RLM model was stratified in accordance with whether the transplant recipient is negative for the KIR ligand (C1, C2, and/or Bw4 epitopes on HLA class I) for the respective iKIR present in the donor (KIR2DL1, 2DL2, 2DL3, and/or 3DL1). Transplants were defined as KIR receptor-ligand mismatched if the donor possessed at least one iKIR that did not recognise the recipient's KIR ligands, or KIR receptor-ligand matched if the recipient possessed all the cognate ligands for the donor's iKIR (Leung et al., 2004). The absence of any iKIR/ligand compatibility combination was defined as receptor/ligand mismatched.

Non-relapse mortality was defined as mortality where relapse, disease progression, or secondary malignancy was not noted as the primary cause of death. End-point events in event free survival (EFS) and event hazard ratio (HR) measurement were defined as all-cause mortality, relapse or secondary malignancy, or re-transplantation. Graft versus Host Disease (GvHD) grading was based on clinical notes. Where GvHD grading definition was unavailable in the recipient's medical notes, the prescription of steroids in treatment of GvHD was accepted as equivalence to GvHD grade II or above. Complete Remission (CR) status, cytogenetic risk levels, and conditioning regime classification were also based upon clinical notes. CMV serostatus was defined by CMV immunoglobulin testing carried out pre-transplant, with the same CMV serostatus results in both donor and recipient described as CMV serostatus matched, and different serostatus between donor and recipient in either direction described as CMV serostatus mismatched.

4.2.5 Ethics

Ethical approval was obtained from the NHS Health Research Authority and Health and Care Research Wales (https://www.hra.nhs.uk/, application number: REC: 18/NW/0553, Appendix B.iii). The study was authorised by the Manchester University NHS Foundation Trust Clinical Trials Management Offices. Informed consent for use of residual genetic material in research relating to transplantation was granted by all transplant recipients and donation at the point of consenting to the transplant.

4.3 Results

4.3.1 Transplant Characteristics

This study investigated 115 transplants given to AML patients from a single centre, performed between 1st October 2011 and 31st March 2016. A subgroup of the cohort, T cell depleted reduced intensity conditioning (RIC) transplants (n=88), were analysed in parallel with the full cohort (n=115). Transplant pair demographic data can be found in Table 22. The cohort included patients with a wide range of disease stage and risk levels, with 73.0% of recipients transplanted in CR1 and 53.0% presenting as standard risk by cytogenetics. A majority of transplants carried out were 10/10 HLA allele matched at HLA-A, B, C, DRB1, and DQB1, with RIC and T cell depletion, using a peripheral blood progenitor cell (PBPC) source (60.9%). Cohorts A and B presented comparable proportions in demographic data, with the exception of clinical transplant work-up.

All following data describes Cohort B (T cell depleted RIC transplants, unless otherwise stated). The data relating to Cohort A can be found in Appendix A.i.

A majority of donors possessed KIR B/x diplotypes (73.9%). In accordance with the B content scoring model, a majority of the transplant donors were categorised as Neutral (70.5%), with smaller proportions categorised as Better (21.6%) and Best (8.0%) (Cooley et al., 2010). 44.3% of transplant donors were both KIR B/x and classified as Neutral in KIR B content. 65.9% of transplants were KIR mismatched as defined by the KIR RLM model (Leung et al., 2004). Comparable proportions of recipients were C1/C1 epitope homozygous or C1/C2 epitope heterozygous (45.5% and 43.2% respectively), with a lesser frequency C2/C2 epitope homozygous (11.4%). KIR characteristics were analogous between cohorts A and B.

Variable	Cohort A (n=115)	Cohort B (n=88)	Variable	Cohort A (n=115)	Cohort B (n=88)
Median Age (Range)			**Conditioning Regime**		
Recipient	56.0 yrs median (25-75 yrs)	57.5 yrs median (25-72 yrs)	Reduced Intensity (RIC)	100 (87.0%)	88 (100%)
Donor	36.0 yrs median (18-68 yrs)	36.5 yrs median (18-68 yrs)	Myeloablative (MAC)	15 (13.0%)	-
Recipient/Donor Sex			**Conditioning Protocol**		
Recip. Female/ Donor Female	22 (19.1%)	20 (22.7%)	Flu + Melph	56 (48.7%)	56 (63.6%)
Recip. Male/ Donor Female	14 (12.2%)	10 (11.4%)	Flu/Bu	22 (19.1%)	22 (25.0%)
Recip. Female/ Donor Male	19 (16.5%)	14 (15.9%)	Bu + Cy	2 (1.7%)	-
Recip. Male/ Donor Male	60 (52.2%)	44 (50.0%)	Flu/Bu + ARA-C + AMSA	2 (1.7%)	2 (2.3%)
Recipient/Donor HLA match (HLA-A, B, C, DRB1, and DQB1)			TBI + Flu	15 (13.0%)	2 (2.3%)
10/10 allele matched	93 (80.9%)	71 (80.7%)	TBI + Cy	12 (10.4%)	-
9/10 allele matched	22 (19.1%)	17 (19.3%)	TBI + Flu + Cy	1 (0.9%)	1 (1.1%)
HLA-A mismatch	*4 (3.5%)*	*3 (3.4%)*	TBI + Flu + ARA-C + AMSA + Cy	5 (4.3%)	5 (56.8%)
HLA-B mismatch	*2 (1.7%)*	*2 (2.3%)*			
HLA-C mismatch	*9 (7.8%)*	*8 (9.1%)*			
HLA-DQB1 mismatch	*7 (6.1%)*	*4 (4.5%)*			
Donor Cell Source			**T cell Depletion**		
Peripheral Blood Progenitor Cells	111 (96.5%)	86 (97.7%)	Alem	67 (58.3%)	56 (63.6%)
Bone Marrow	4 (3.5%)	2 (2.3%)	ATG	32 (27.8%)	32 (36.4%)
			T cell replete	16 (13.9%)	-
CMV Serostatus			**Disease Risk Level by Cytogenetics**		
Recipient Neg / Donor Neg	40 (37.8%)	27 (30.7%)	Good	1 (0.9%)	1 (1.1%)
Recipient Pos / Donor Pos	53 (46.1%)	45 (51.1%)	Standard	61 (53.0%)	49 (55.7%)
Recipient Pos / Donor Neg	12 (10.4%)	8 (9.1%)	Intermediate	2 (1.7%)	1 (1.1%)
Recipient Neg / Donor Pos	8 (7.0%)	7 (8.0%)	Poor	46 (40.0%)	35 (39.8%)
Recipient Equivocal / Donor Neg	1 (0.9%)	1 (1.1%)	Data Unavailable	5 (4.3%)	2 (2.3%)
Recipient Unknown / Donor Neg	1 (0.9%)	-			
CR Status			**GvHD Prophylaxis**		
1	84 (73.0%)	63 (71.6%)	CSA	72 (62.6%)	62 (70.5%)
2	29 (25.2%)	23 (26.1%)	CSA + Meth	22 (26.1%)	17 (19.3%)
3	1 (0.9%)	1 (1.1%)	CSA + MMF	4 (3.5%)	4 (4.5%)
Data Unavailable	1 (0.9%)	1 (1.1%)	MMF	3 (2.6%)	3 (3.4%)
			CSA + Meth + MMF	14 (12.2%)	2 (2.3%)

Table 22 – Cohort Demographics Data Descriptive statistics of the clinical data and medical protocols used, for cohorts A and B.

Key: *Alem: Alemtuzumab (Campath). ATG: Anti-Thymocyte Globulin. Cy: Cyclophosphamide. CSA: Cyclosporin A. Meth: Methotrexate. MMF: Mycophenolate mofetil. Flu: Fludarabine. Melph: Melphalan. Bu: Busulfan. ARA-C: Cytarabine. AMSA: Amsacrine. TBI: Total Body Irradiation.*

A number of non-KIR characteristics were investigated for their influence upon transplant outcomes by univariate analysis (Table 23). The only characteristic to reach significance was donor age, with donors ≥30yrs presenting increased risk of all-cause mortality (HR=2.20, 95% CI=1.12-4.33, p=0.023, p_c=0.115) and non-relapse mortality (NRM) (HR=7.94, 95% CI=1.03-61.14, p=0.047, p_c=0.188) within the initial 3 years post-transplant. No other factor provided significant influence for the outcomes investigated, including 9/10 versus 10/10 HLA matching (to second field resolution), CMV serostatus matching, conditioning regime, donor type (HLA matched sibling (mSib) or HLA matched unrelated donor (MUD)), patient disease risk group and CR status. Cohort A displayed a trend towards transplants using donors ≥30yrs also increasing risk of GvHD risk II-IV (HR=1.80, 95% CI=0.94-3.46, p=0.077, p_c=0.231), but this effect was not found in Cohort B (p=0.402).

Factor	Group	Category	n (%)	Mortality HR (95% CI)	Non-Relapse Mortality HR (95% CI)	Event HR (95% CI)	Relapse HR (95% CI)	GvHD grade II-IV HR (95% CI)
Recipient Sex	Cohort A (All Transplants)	Male Female	74 (64.3%) 41 (35.7%)	1 1.43 (0.84-2.42) (p=0.185)	1 0.96 (0.36-2.55) (p=0.956)	1 1.40 (0.85-2.31) (p=0.183)	1 1.26 (0.65-2.45) (p=0.498)	1 1.16 (0.62-2.15) (p=0.643)
	Cohort B (T cell Depleted RIC Transplants)	Male Female	54 (61.4%) 34 (38.6%)	1 1.18 (0.65-2.12) (p=0.587)	1 0.50 (0.14-1.83) (p=0.502)	1 1.23 (0.70-2.15) (p=0.468)	1 1.12 (0.54-2.31) (p=0.759)	1 0.92 (0.47-1.96) (p=0.917)
Donor Sex	Cohort A	Male Female	79 (68.7%) 36 (31.3%)	1 1.22 (0.71-2.10) (p=0.476)	1 0.81 (0.42-3.02) (p=0.806)	1 1.12 (0.66-1.88) (p=0.681)	1 1.23 (0.63-2.42) (p=0.543)	1 1.09 (0.58-2.06) (p=0.791)
	Cohort B	Male Female	58 (65.9%) 30 (34.1%)	1 1.32 (0.73-2.39) (p=0.356)	1 0.91 (0.28-2.95) (p=0.870)	1 1.15 (0.65-2.05) (p=0.632)	1 1.52 (0.74-3.10) (p=0.254)	1 0.95 (0.46-1.95) (p=0.883)
Donor Age	Cohort A	Donor <30yrs Donor ≥30yrs	43 (37.4%) 72 (62.6%)	1 2.07 (1.15-3.73) (p=0.016, p_c=0.080)	1 5.78 (1.33-25.16) (p=0.019, p_c=0.080)	1 1.42 (0.84-2.39) (p=0.191)	1 0.91 (0.47-1.73) (p=0.763)	1 1.80 (0.94-3.46) (p=0.077, p_c=0.231)
	Cohort B	Donor <30yrs Donor ≥30yrs	31 (35.2%) 57 (64.8%)	1 2.20 (1.12-4.33) (p=0.023, p_c=0.115)	1 7.94 (1.03-61.14) (p=0.047, p_c=0.188)	1 1.41 (0.78-2.54) (p=0.259)	1 0.89 (0.44-1.82) (p=0.752)	1 1.36 (0.66-2.81) (p=0.402)
Recipient/ Donor HLA match	Cohort A	10/10 HLA match 9/10 HLA match	93 (80.9%) 22 (19.1%)	1 1.18 (0.63-2.24) (p=0.607)	1 1.29 (0.42-3.92) (p=0.658)	1 1.26 (0.70-2.28) (p=0.446)	1 1.64 (0.79-3.38) (p=0.184)	1 0.86 ().38-1.92) (p=0.709)
	Cohort B	10/10 HLA match 9/10 HLA match	71 (80.7%) 17 (19.3%)	1 1.15 (0.57-2.32) (p=0.694)	1 0.97 (0.40-2.36) (p=0.949)	1 1.00 (0.50-2.00) (p=0.997)	1 1.18 (0.51-2.75) (p=0.694)	1 0.97 (0.40-2.36) (p=0.949)
CMV	Cohort A	Matched Mismatched Data Unavailable	93 (80.9%) 20 (17.4%) 2 (1.7%)	1 1.17 (0.60-2.25) (p=0.650) -	1 1.02 (0.29-3.54) (p=0.981) -	1 1.01 (0.53-1.93) (p=0.986) -	1 1.12 (0.49-2.54) (p=0.793) -	1 0.43 (0.15-1.20) (p=0.105) -
	Cohort B	Matched Mismatched Data Unavailable	71 (80.7%) 16 (18.2%) 1 (1.1%)	1 1.12 (0.54-2.32) (p=0.759) -	1 1.34 (0.37-4.88) (p=0.654) -	1 0.94 (0.46-1.92) (p=0.854) -	1 0.84 (0.32-2.20) (p=0.728) -	1 0.38 (0.12-1.24) (p=0.109) -
Disease Risk Level	Cohort A	Good Standard Intermediate/Poor Data Unavailable	1 (0.9%) 61 (53.0%) 48 (41.7%) 5 (4.3%)	- 1 0.99 (0.58-1.69) (p=0.967) -	- 1 0.56 (0.20-1.62) (p=0.285) -	- 1 0.95 (0.57-1.59) (p=0.852) -	- 1 1.19 (0.61-2.31) (p=0.606) -	- 1 0.85 (0.45-1.61) (p=0.611) -
	Cohort B	Good Standard Intermediate/Poor Data Unavailable	1 (1.1%) 49 (55.7%) 36 (40.9%) 2 (2.3%)	- 1 1.17 (0.64-2.13) (p=0.605) -	- 1 0.50 (0.13-1.9) (p=0.312) -	- 1 1.14 (0.64-2.01) (p=0.648) -	- 1 1.34 (0.66-2.75) (p=0.419) -	- 1 0.85 (0.41-1.76) (p=0.654) -
CR Status	Cohort A	1 ≥2 Data Unavailable	84 (73.0%) 30 (26.1%) 1 (0.9%)	1 1.53 (0.88-2.66) (p=0.130) -	1 1.91 (0.74-4.93) (p=0.181) -	1 1.37 (0.81-2.33) (p=0.243) -	1 0.97 (0.46-2.05) (p=0.934) -	1 1.53 (0.80-2.95) (p=0.202) -
	Cohort B	1 ≥2 Data Unavailable	63 (71.6%) 24(27.3%) 1 (1.1%)	1 1.35 (0.731-2.51) (p=0.336) -	1 1.67 (0.55-5.11) (p=0.369) -	1 1.25 (0.69-2.25) (p=0.464) -	1 0.95 (0.43-2.13) (p=0.907) -	1 1.40 (0.66-2.96) (p=0.380) -
Donor Type	Cohort A	HLA matched Sibling MUD	30 (26.1%) 85 (73.9%)	1 0.95 (0.52-1.71) (p=0.853)	1 1.18 (0.39-3.59) (p=0.768)	1 1.14 (0.64-2.04) (p=0.652)	1 1.24 (0.57-2.72) (p=0.589)	1 0.93 (0.47-1.85) (p=0.843)
	Cohort B	HLA matched Sibling MUD	24 (27.3%) 64 (72.7%)	1 0.75 (0.40-1.41) (p=0.375)	1 1.11 (0.30-4.0) (p=0.877)	1 0.90 (0.48-1.66) (p=0.726)	1 0.96 (0.43-2.16) (p=0.928)	1 1.14 (0.52-2.54) (p=0.742)
Conditioning Regime	Cohort A	Reduced Intensity (RIC) Myeloablative (MAC)	100 (87.0%) 15 (13.0%)	1 0.68 (0.27-1.71) (p=0.415)	1 0.42 (0.06-3.15) (p=0.397)	1 0.88 (0.40-1.93) (p=0.747)	1 1.11 (0.43-2.85) (p=0.827)	1 0.66 (0.23-1.84) (p=0.421)

Table 23 – Univariate analysis of non-KIR characteristics in Cohort A (n=115) and Cohort B (n=88).

Conditioning regime is only presented for Cohort A, as all transplants within Cohort B used reduced intensity conditioning (RIC).

Key: Data with p≤0.05 are shaded blue. Data with 0.05<p≤0.10 are shaded orange.

4.3.2 KIR & Transplant Outcome

Table 24 contains a summary of the findings for both Cohorts A and B. Kaplan Meier curves for the Cohort A data can be found in the Appendix A.ii.

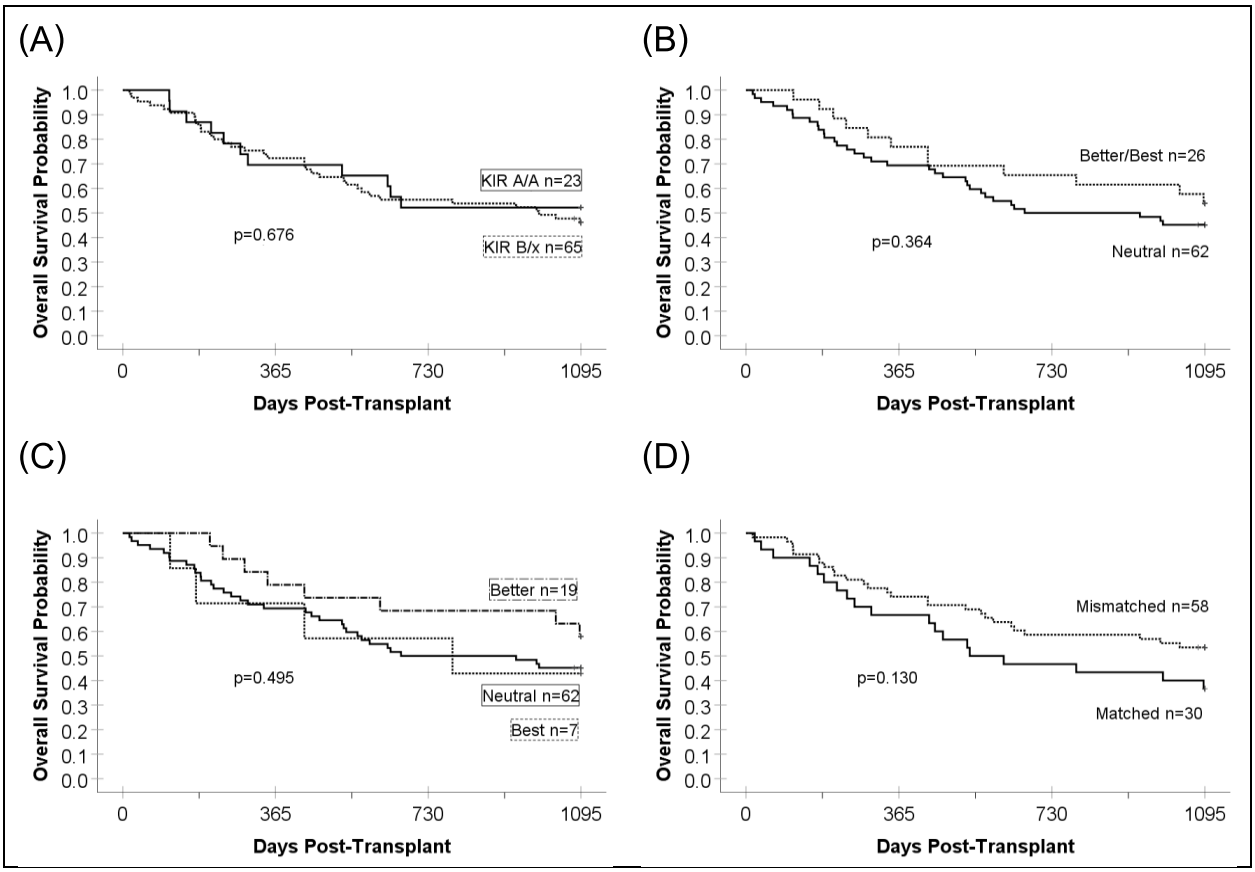


Figure 22 – Comparison of Overall Survival (OS) between investigated KIR assessment models in T cell depleted RIC HPCT (Cohort B) (n=88).

(A) Donor KIR A/A versus B/x diplotypes, (B) KIR B Content Scoring Model (grouping Better and Best) (Cooley et al., 2010), (C) B Content Scoring Model (Cooley et al., 2010), (D) KIR Receptor/Ligand Mismatching Model (Leung et al., 2004).

4.3.3 Survival Post-Transplant

No KIR assessment model reached significance in predicting overall survival (OS) (Table 24, Figure 22). The RLM model displayed some divergence of strata in the survival curve, with RLM mismatched transplants presenting improved survival compared to RLM matched transplants, but this did not reach significance (53.4% versus 36.7%, p=0.130). Donors ranked Better or Best by the B Content Scoring

model presented a nominal non-significant improvement compared to Neutral donors (53.9% versus 45.2%, p=0.364).

KIR Assessment Model	Cohort	Category	n (%)	Overall Mortality HR (95% CI)	Non-Relapse Mortality HR (95% CI)	Event HR (95% CI)	Relapse HR (95% CI)	GvHD grade II-IV HR (95% CI)
Donor KIR A/A versus B/x Diplotypes	Cohort A (All Transplants)	KIR A/A KIR B/x	34 (30.0%) 81 (70.4%)	1 1.05 (0.59-1.88) (p=0.863)	1 2.04 (0.59-7.04) (p=0.261)	1 1.07 (0.620-1.85) (p=0.807)	1 0.765 (0.39-1.50) (p=0.765)	1 2.13 (0.99-4.60) (p=0.054, p_c=0.270)
	Cohort B (T cell Depleted RIC Transplants)	KIR A/A KIR B/x	23 (26.1%) 65 (73.9%)	1 1.16 (0.59-2.28) (p=0.676)	1 32.29 (0.17-6208.95) (p=0.195)	1 1.18 (0.62-2.25) (p=0.624)	1 0.75 (0.35-1.60) (p=0.457)	1 3.22 (1.13-9.17) (p=0.029, p_c=0.145)
KIR B Content Scoring Model (Better and Best grouped) (2010)	Cohort A	Neutral Better/Best	82 (71.3%) 33 (28.7%)	1 0.87 (0.49-1.54) (p=0.627)	1 1.19 (0.42-2.98) (p=0.822)	1 0.89 (0.51-1.53) (p=0.666)	1 0.62 (0.284-1.36) (p=0.235)	1 1.68 (0.91-3.07) (p=0.095, p_c=0.475)
	Cohort B	Neutral Better/Best	62 (70.5%) 26 (29.5%)	1 0.74 (0.38-1.43) (p=0.366)	1 0.63 (0.27-1.46) (p=0.281)	1 0.82 (0.44-1.51) (p=0.520)	1 0.63 (0.27-1.46) (p=0.281)	1 0.80 (0.57-1.13) (p=0.210)
KIR B Content Scoring Model (2010)	Cohort A	Neutral Better Best	82 (71.3%) 23 (20.0%) 10 (8.7%)	1 (p=0.819) 0.81 (0.42-1.58) (p=0.538) 1.02 (0.40-2.58) (p=0.968)	1 (p=0.911) 1.02 (0.33-3.16) (p=0.973) 1.39 (0.31-6.21) (p=0.667)	1 (p=0.911) 0.89 (0.48-1.65) (p=0.707) 0.88 (0.35-2.22) (p=0.789)	1 (0.439) 0.54 (0.21-1.39) (p=0.202) 0.84 (0.26-2.75) (p=0.837)	1 (p=0.082) 2.02 (1.07-3.83) (p=0.031, p_c=0.155) 0.90 (0.27-2.99) (p=0.868)
	Cohort B	Neutral Better Best	62 (70.5%) 19 (21.6%) 7 (8.0%)	1 (p=0.501) 0.64 (0.30-1.38) (p=0.255) 1.07 (0.38-3.01) (p=0.900)	1 (p=0.495) 0.63 (0.14-2.91) (p=0.551) 2.02 (0.44-9.36) (p=0.368)	1 (p=0.778) 0.78 (0.39-1.56) (p=0.478) 0.94 (0.34-2.64) (p=0.905)	1 (p=0.550) 0.60 (0.23-1.57) (p=0.296) 0.73 (0.17-3.07) (p=0.662)	1 (p=0.264) 1.78 (0.86-3.67) (p=0.118) 0.89 (0.21-3.80) (p=0.870)
KIR Receptor/Ligand Mismatching Model	Cohort A	Matched Mismatched	38 (33.0%) 77 (67.0%)	1 0.67 (0.39-1.14) (p=0.143)	1 0.42 (0.17-1.08) (p=0.072, p_c=0.360)	1 0.70 (0.42-1.16) (p=0.165)	1 1.15 (0.55-2.37) (p=0.713)	1 1.43 (0.72-2.84) (p=0.306)
	Cohort B	Matched Mismatched	30 (34.1%) 58 (65.9%)	1 0.64 (0.35-1.15) (p=0.134)	1 0.40 (0.13-1.19) (p=0.098, p_c=0.490)	1 0.68 (0.39-1.20) (p=0.186)	1 1.08 (0.50-2.35) (p=0.843)	1 1.42 (0.66-3.06) (p=0.370)

Table 24 – Comparison of KIR assessment models in transplantation in Cohort A (n=115) and Cohort B (n=88).

Key: Data with p≤0.05 are shaded blue. Data with 0.05<p≤0.10 are shaded orange.

Cohort A presented similar findings to those of Cohort B, with no significant improvement in OS found with any of the investigated models. No improvement was observed in Better or Best donor transplants by the B Content Scoring model (51.1% versus 49.1%, p=0.627).

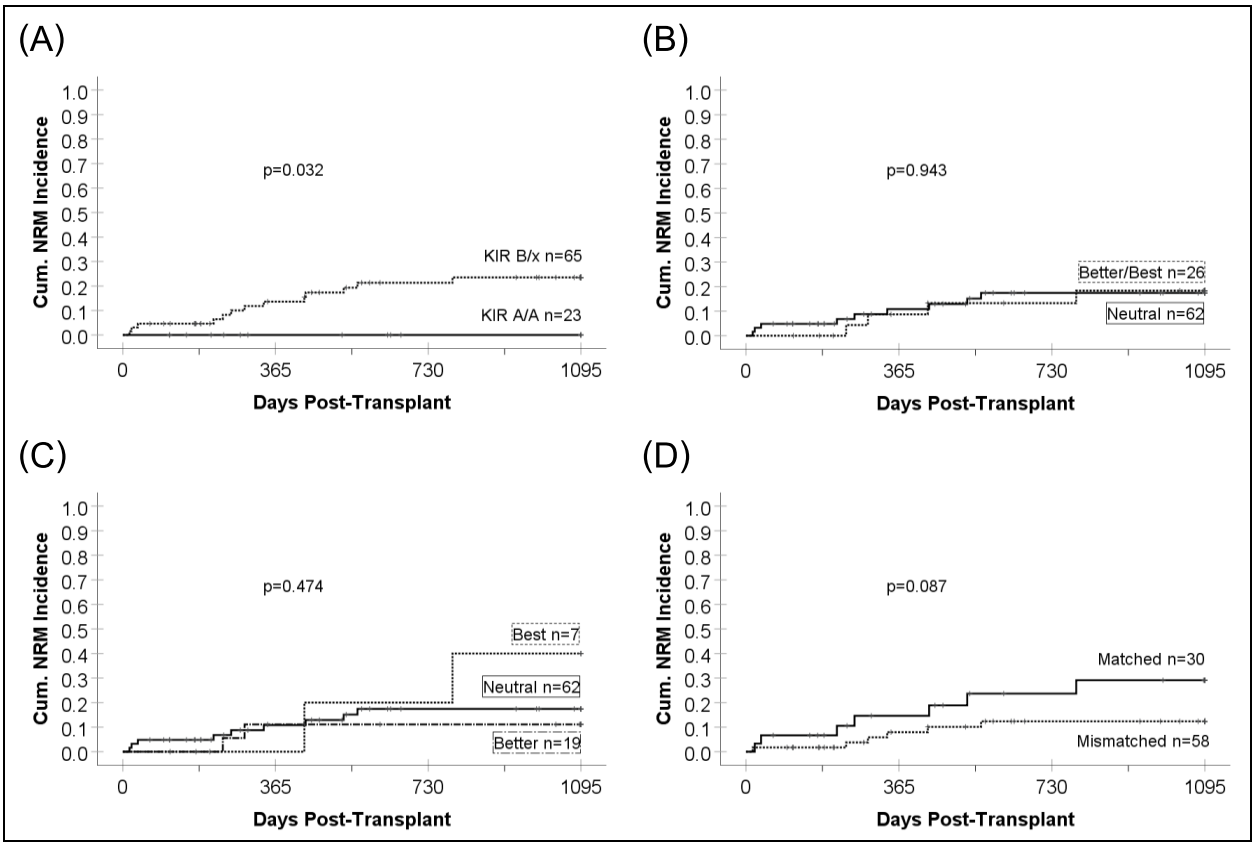


Figure 23 – Comparison of non-relapse mortality (NRM) between investigated KIR assessment models in T cell depleted RIC HPCT (Cohort B) (n=88).

(A) Donor KIR A/A versus B/x diplotypes (p=0.032, p_c=0.128), (B) KIR B Content Scoring Model (combining Better and Best) (p=0.943) (Cooley et al., 2010), (C) B Content Scoring Model (p=0.474) (Cooley et al., 2010), (D) KIR Receptor/Ligand Mismatching Model (Leung et al., 2004) (p=0.087, p_c=0.261).

Transplants using donors with KIR A/A diplotypes presented a significant improvement in 3 years NRM (0.00% versus 23.5%, p=0.032, p_c=0.128), but this did not provide a significant hazard ratio (HR=32.29, 95% CI=0.17-6208.95, p=0.195). The KIR RLM model trended towards receptor/ligand mismatched transplants improving 3-year NRM rates for KIR mismatched transplants (29.1% versus 12.3%, p=0.087, p_c=0.261; HR=0.40, 95% CI=0.13-1.19, p=0.098, p_c=0.490). These

observations did not reach significance. No other model investigated reduced risk of NRM.

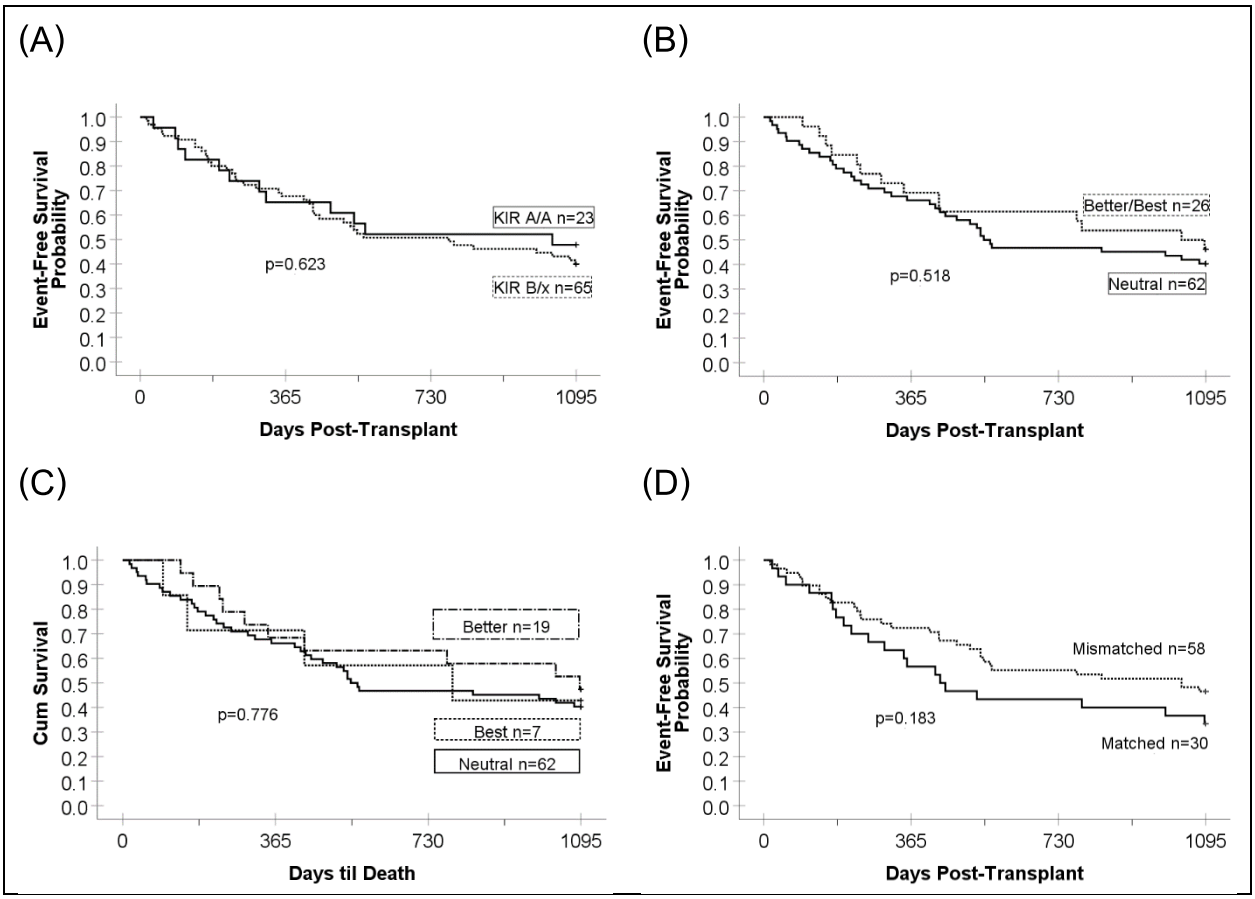


Figure 24 – Comparison of event-free survival (EFS) between investigated KIR assessment models in T cell depleted RIC HPCT (Cohort B) (n=88).

(A) Donor KIR A/A versus B/x diplotypes (p=0.623), (B) KIR B Content Scoring Model (combining Better and Best) (p=0.518) (Cooley et al., 2010), (C) B Content Scoring Model (p=0.776) (Cooley et al., 2010), (D) KIR Receptor/Ligand Mismatching Model (p=0.183) (Leung et al., 2004).

In the Cohort A, the improvement in 3-year NRM for transplants with KIR A/A diplotype donors was greatly reduced (11.5 KIR A/A donor versus 22.7% KIR B/x donor, p=0.251) compared to that of Cohort B (Figure 23). The RLM model presented a similar non-significant improvement in NRM in all transplants compared to the T cell depleted RIC transplant cohort (14.6% versus 30.2%, p=0.064, p_c=0.256; HR=0.40, 95% CI=0.17-1.08, p=0.072, p_c=0.360).

Receptor/ligand mismatched transplants provided a nominal non-significant improvement in 3-year EFS compared to matched transplants (46.6% versus 33.3%, p=0.183) (Figure 24). Similar observations were found in Cohort A. No other finding concerning EFS approached significance.

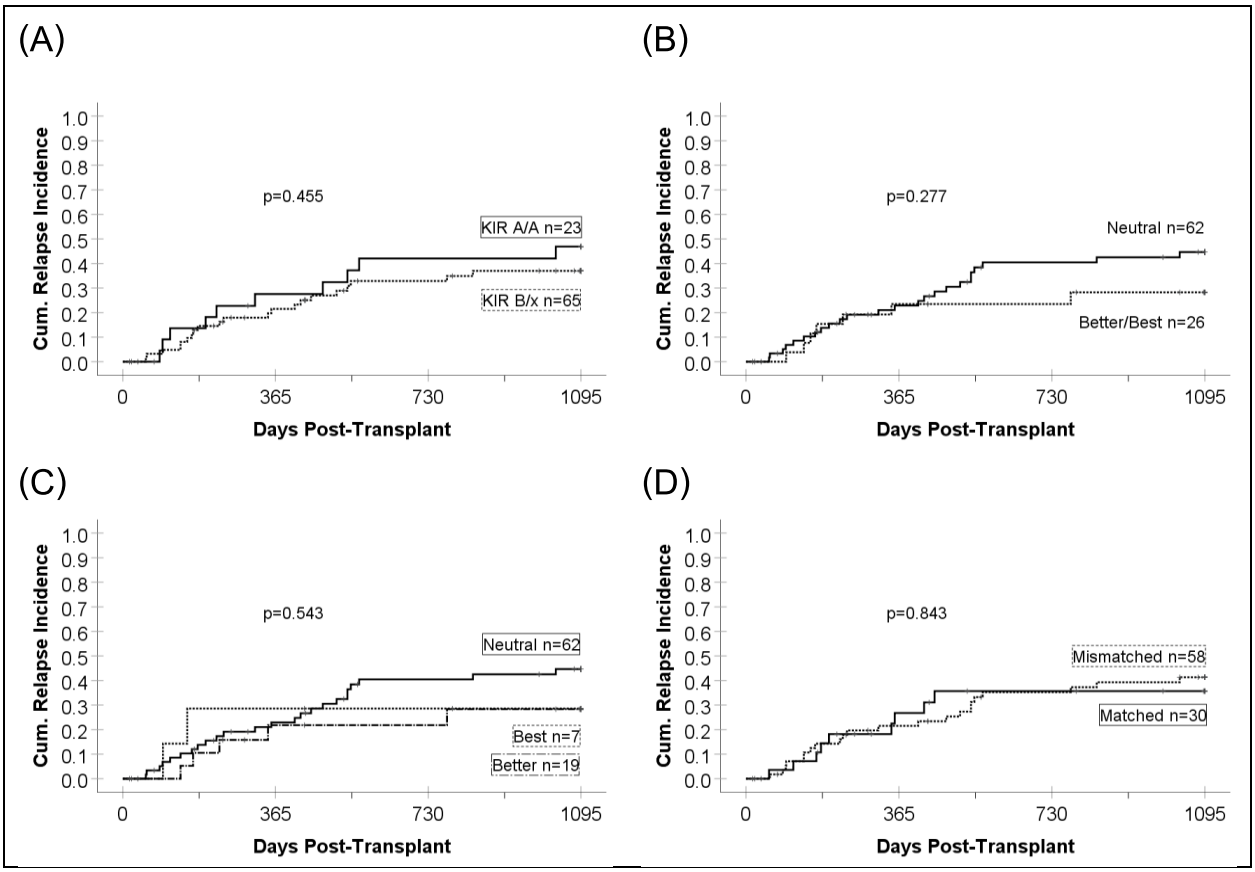


Figure 25 – Comparison of relapse between investigated KIR assessment models in T cell depleted RIC HPCT (Cohort B) (n=88). (A) Donor KIR A/A versus B/x diplotype (p=0.455), (B) KIR B Content Scoring Model (combining Better and Best) (p=0.277) (Cooley et al., 2010), (C) B Content Scoring Model (p=0.543) (Cooley et al., 2010), (D) KIR Receptor/Ligand Mismatching Model (p=0.843) (Leung et al., 2004).

4.3.4 Relapse

No investigated algorithm improved relapse rates (Figure 25). Donors with KIR gene content classified Better or Best by the KIR B content scoring model presented a modest non-significant improvement in 3-year relapse (28.3 versus 44.6%, p=0.277). This divergence is apparent after the initial year post-transplant, with both strata presenting 23.5% relapse at 1-year post-transplant, with the groups diverging at this point. Similar effects in 3-year relapse incidence were observed in Cohort A (40.8% Neutral donors versus 25.5% Better/Best donors, p=0.230), with both strata presenting approximately 22% at 1-year post-transplant (Better/Best=22.5%, Neutral=21.6%).

4.3.5 Graft versus Host Disease

Transplants using donors with KIR B/x diplotypes displayed a significantly increased incidence of 3-year GvHD grade II-IV compared to transplants using donors with KIR A/A diplotypes (53.2% versus 19.4%, p=0.021, p_c=0.084; HR=3.22, 95% CI=1.13-9.17, p=0.029, p_c=0.145). The KIR B content scoring model presented a non-significant divergence in outcome, with transplants using donors classified as Better experiencing an increased incidence of GvHD grade II-IV compared to donors classified as Neutral or Best (65.9% versus 35.7% versus 35.7% respectively, p=0.254; Better versus Neutral HR=1.78, 95% CI=0.86-3.67, p=0.118). The KIR RLM model presented no influence upon incidence of GvHD grade II-IV.

Cohort A presented a stronger correlation between the use of donors with Better classification (in accordance to the B content scoring model) and the recipient experiencing greater risk of GvHD grade II-IV (Better versus Neutral HR= 2.02, 95% CI=1.07-3.83, p=0.031) (Table 24, Figure 26). 69.5% of transplants using donors classified as Better experienced GvHD grade II-IV by 3-years post-transplant, in

contrast to 40.0% of transplants using donors classified as Best, and 37.2% of transplants using donors classified as Neutral (p=0.074, p_c=0.222). In comparison to the T cell depleted RIC transplants cohort, there was a weaker trend towards transplants using KIR B/x diplotype donors experiencing increased risk of GvHD grade II-IV within 3 years post-transplant compared to A/A diplotype donors (52.7% versus 29.3%, p=0.048, p_c=0.192; HR=2.13, 95% CI=0.99-4.60, p=0.054).

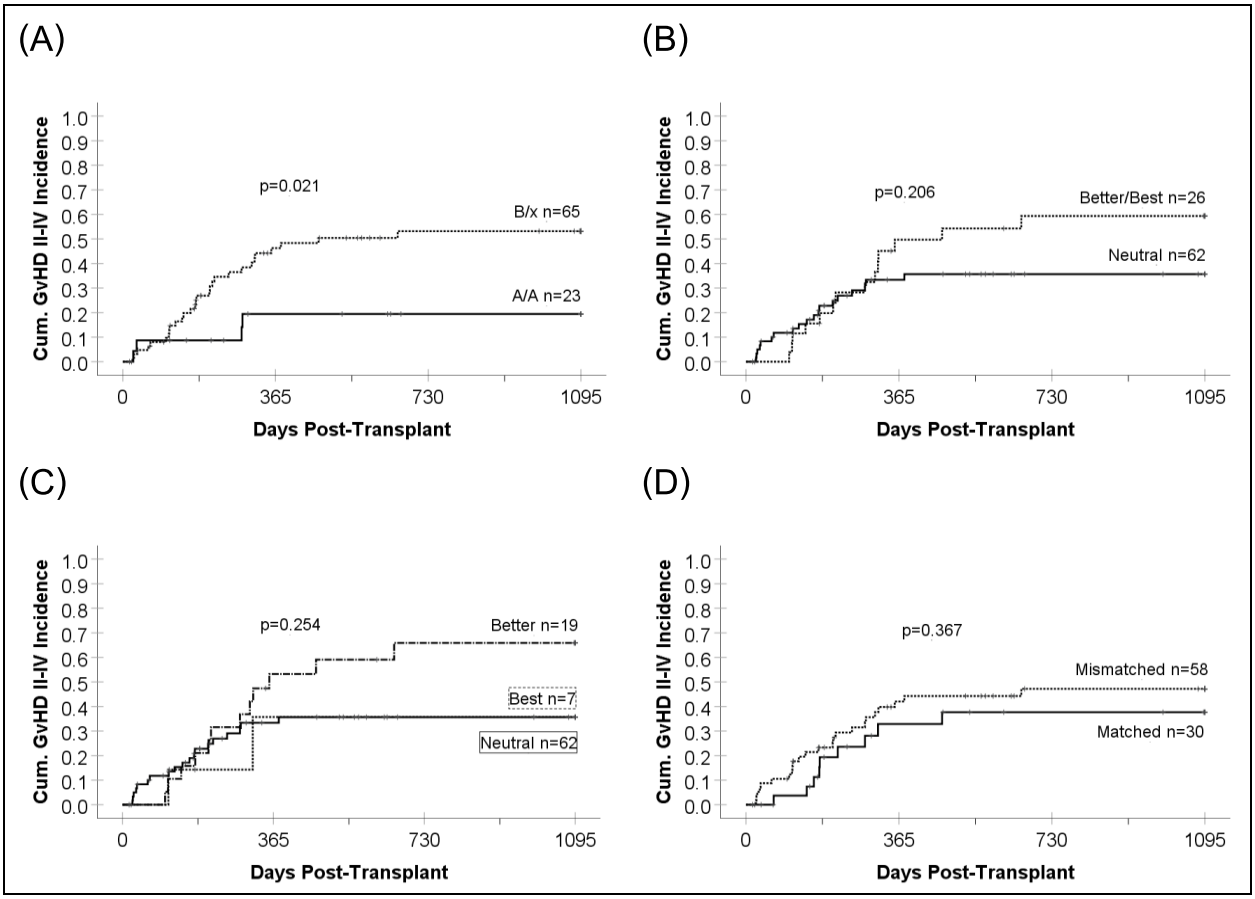


Figure 26 – Comparison of graft versus host disease (GvHD) between investigated KIR assessment models in T cell depleted RIC HPCT (n=88).

(A) Donor KIR A/A versus B/x diplotypes (p=0.021, p_c=0.084), (B) KIR B Content Scoring Model (combining Better and Best) (p=0.206) (Cooley et al., 2010), (C) B Content Scoring Model (p=0.254) (Cooley et al., 2010), (D) KIR Receptor/Ligand Mismatching Model (p=0.367) (Leung et al., 2004).

4.4 Discussion

This study examined the relative predictive value of several published models for assessing donor KIR diplotypes in a single centre cohort of 115 HPCTs in treatment of AML, dated 2011 to 2016. The cohort was analysed in two groupings: all transplants (Cohort A, n=115) and T cell depleted RIC transplants (Cohort B, n=88). Four distinct models were assessed: KIR A/A versus B/x diplotype donor transplants, the RLM model, and two forms of the B content scoring model (2010) (Leung et al., 2004; Cooley et al., 2010). The B content scoring model was assessed in its published form (three groups: Neutral, Better and Best), and with the Better and Best groups combined (i.e. B score ≥2, without weighting for KIR CenB regions), due to the low number of Best donor transplants in the cohort (n=7 in Cohort B).

Both cohorts A and B presented similar findings in many outcomes, partially owing to T cell depleted RIC transplants representing a majority of the all transplants cohort. No model investigated correlated with improved OS, EFS or relapse. KIR B/x diplotypes donor transplants presented a stronger predictor for the risk of developing GvHD grade II-IV in both cohorts. KIR receptor/ligand mismatched transplants trended towards reducing NRM incidence at 3 years post-transplant, with a similar trend demonstrated in both cohorts (Cohort A: 14.6% versus 30.2%, p=0.064; Cohort B: 12.3% versus 29.1%, p=0.087).

The study did not verify the original published findings of the respective clinical models. The RLM model proposes to reduce 3-year relapse incidence in KIR receptor/ligand mismatched transplants (Leung et al., 2004). The B content scoring model similarly describes a reduction in 3-year relapse incidence in donors ranked ‘better’ or ‘best’ (Cooley et al., 2010). In this study, a nominal improvement was

observed in ‘better’ and ‘best’ donors compared to ‘neutral’ donors (in accordance with the B content scoring model), but this did not approach significance (28.3% with better/best donors versus 44.6% with neutral donors, p=0.277). No difference was observed in 3-year relapse incidence between receptor/ligand matched (35.7%) and mismatched transplants (41.3%) (p=0.843). These data do not preclude the described clinical algorithms successfully functioning in other transplant centres, but highlight the importance of validating clinical models prior to implementation to ensure they are suitable for the respective transplant centre. There were profound differences in the populations described in both the RLM model (paediatric sample, mixture of myeloid and lymphoid leukaemia patients) and B content scoring model (T cell replete, myeloablative conditioning) publications compared to the cohort of this study, which may explain why the results were not reproducible in this setting.

It would be expected that any significant finding that was relevant to T cell depletion and RIC would be strengthened upon filtering for these characteristics. One of the greatest differences between the two cohorts was observed in NRM. KIR B/x diplotype donor transplants experienced a higher incidence of 3-year NRM compared to transplants using KIR A/A diplotype donors at 3 years post-transplant in Cohort B (0% versus 23.5% NRM survival for KIR A/A and B/x diplotypes respectively, p=0.032). The RLM model did not significantly influence incidence of NRM Cohort B (11.5% versus 22.7% for matched and mismatch transplants respectively, p=0.251). This indicates conditioning regimes do have implications for KIR assessment algorithms within donor selection criteria.

The value of KIR donor assessment models in GvHD grade II-IV also varied between the two cohorts. KIR B/x donor transplants increased risk of GvHD grade II-IV in the

first 3 years post-transplant, with this model providing a stronger predictor in Cohort B (HR=3.22, 95% CI=1.13-9.17, p=0.029, p_c=0.145) compared to Cohort A (HR=2.13, 95% CI=0.99-4.60, p=0.054, p_c=0.270). The B content scoring model trended towards Better/Best donor transplants experiencing a higher risk of GvHD grade II-IV in Cohort A (HR=1.68, 95% CI=0.91-3.07, p=0.095, p_c=0.475). This model did not provide significance in Cohort B, likely due to the smaller cohort size (p=0.210).

The results of this study contribute to the mounting evidence that KIR assessment models in HPCT donor selection influence the incidence NRM and GvHD grade II-IV, suggesting they may be indicative of wider implications. Of the thirteen patients to die due to NRM within the first 3 years post-transplant (all receiving KIR B/x diplotype donations), nine (69.2%) reported infection as the primary or secondary cause of death: six listed respiratory infection as the sole cause of death (four cases of pneumonia, one case of Influenza A, and one undefined chest infection), and three deaths noted infection as a contributing factor (pneumonia with severe GvHD, Respiratory Syncytial Virus infection with cardiac arrest, and active CMV infection with pancytopaenia). The remaining deaths were caused by organ failure (three deaths), and cerebral bleed refractory to platelet transfusions (one death). Deaths ranged from 16-561 days post-transplant (median: 291 days). This suggests transplants using donors with KIR A/A diplotypes may offer protection against infection post-transplant. Bultitude et al (2020) identified a similar link in the susceptibility of HPCT using KIR B/x diplotypes to infection mortality in a cohort of T cell depleted MAC transplants, suggesting NK cell immunity in the absence of T cells may be an important component for this protection. As one of the first donor

immune cells to reconstitute post-transplant, NK cells likely play a dominant role in establishing the immunological environment in the recipient (Pical-Izard et al., 2015).

The models compared in this study were selected due to their independence from mismatched KIR ligands (HLA class I) between recipient and donor. HLA class I and II represents the primary selection criteria for donor selection in HPCT between recipient and donor (Petersdorf et al., 2004; Shaw et al., 2017; Mayor et al., 2019). A majority (80.7% of Cohort A, 80.4% of Cohort B) of patients within the examined sample received a 10/10 HLA matched graft at HLA-A, B, C, DRB1, and DQB1, with only 15 transplants in Cohort A (13.2%) and 13 in Cohort B (14.9%) mismatched at HLA class I. Consequently, any KIR assessment model that requires consistent implementation of HLA class I incompatibility between recipient and donor would be ineffectual in conjunction with current clinical practices. Only five of these HLA class I mismatches (38.5% of all HLA class I mismatched transplants) resulted in a dissimilar KIR ligand presence/absence profile between the recipient and the donor: two C1 disparities, two C2 disparities, and one Bw4 disparity (involving HLA-A*24). KIR ligand assessment models are more appropriate within haploidentical HPCT, where up to half the HLA genotype will differ between the recipient and donor, and in umbilical cord unit transplants, where a higher degree of HLA mismatching is considered tolerable but not optimal (Hough et al., 2016; Dehn et al., 2019; Shimoni et al., 2019; Willem et al., 2019). Studies in these clinical settings are similarly contradictory in their findings, possibly explained by a lack of homogeneity in treatment protocols (Shimoni et al., 2019; Willem et al., 2019). These forms of transplant had minimal representation within the defined population of this study, with only one haplo-identical transplant and two cord blood unit transplants carried out for

the defined patient group in the chosen time period, so these transplant pairs were excluded from analysis to minimise heterogeneity within the study cohort.

The sample size available to a single centre study remains a limitation of research into HPCT. New findings supporting the necessity for stricter homogeneity of clinical protocol for the research cohort place additional stresses upon providing a sample size of sufficient statistical power. To design a single centre study that controls for consistency in T cell depletion and conditioning regime, a larger timespan of transplants must be tolerated, which may introduce other inconsistencies in treatment due to the development of clinical protocols over time. The application of the B content scoring model greatly suffered from the cohort size, with only 7 (8.0%) transplants utilising donors classified as Best in Cohort B. The proportional representation of Neutral (70.5%), Better (21.6%), and Best (8.0%) donors in this study's transplant cohort correlates with the frequencies reported from other European populations (Weisdorf et al., 2019).

As the influence of KIR upon transplant outcomes in the context of treatment protocols becomes better comprehended, an understanding for how much heterogeneity in clinical practice can be tolerated for the purposes of clinical research will also be reached. For this study, it was necessary to consider both 9/10 and 10/10 HLA matched transplants to maintain a sufficient sample size. As the primary selection criteria for selecting a suitable donor, HLA mismatching presents profound effects upon the outcome (Petersdorf et al., 2004; Shaw et al., 2017; Mayor et al., 2019). Transplants containing a single HLA mismatch did not demonstrate a inferior outcome to that of fully HLA matched transplants in this study (p=0.694). HLA mismatched transplants represent a minority of the sample (Cohort A: n=22, 19.3%; Cohort B:

n=17, 19.5%;), but their inclusion may be considered to weaken the findings of the study.

The most significant non-KIR influence upon transplant outcomes identified in this study was that of donors <30 years old. The benefits of younger donors are well documented, with many donor registries focusing their strategic efforts upon building a panel of male donors under the age of 30 (Shaw et al., 2018; Mayor et al., 2019; Schmidt et al., 2020). Transplants with donors under 30 years represented a minority of transplants (n=31, 35.2% of cohort) in the investigated cohort. Consequently, controlling for this factor is difficult within this study. The scarcity of transplants using donors under 30 years in the examined cohort characterises the difficulty in identifying the optimal donor for all patients with the available knowledge of donor selection criteria and relevant guidelines (Little et al., 2016). The limited number of suitable donors must remain a consideration for the utility of KIR assessment models, which embody an additional criterion that can only be considered once several donors of otherwise comparable value have been identified. This is particularly true of matched sibling transplants, where few recipients have (a) several HLA matched siblings with (b) discernibly different KIR genotypes (as defined by the respective algorithm used).

The RLM model is particularly difficult to implement in donor selection, as the KIR epitope (HLA class I) presents more extensive diversity in its presence or absence than the KIR receptor itself (Chapter 3). A large majority of the donors within the cohort possessed the respective iKIR (KIR2DL1: n=84, 95.5% of cohort; KIR2DL2/2DL3: n=88, 100% of cohort; KIR3DL1: n=85, 96.6% of cohort). Consequently, the RLM model assessment of KIR receptor/ligand compatibility primarily evaluates the

recipient's HLA genotype, and so this model is most appropriate for high HLA disparity between recipient and donor, such as in the selection of haplo-identical donors or umbilical cord blood units. This study lacks the sample size to recommend selecting a donor that would otherwise be considered inferior in accordance with existing donor selection guidelines, for example, selecting an HLA mismatched donor over a HLA matched donor (Little et al., 2016). In HLA matched donors, the RLM model may provide a means of identifying recipients more prone to certain outcomes post-transplant, with appropriate amendments to treatment protocols in response to these identified advantages or disadvantages.

Despite selecting transplants from a single centre and within a limited timespan to control for variations in treatment protocols, the studied population presented heterogenous characteristics in treatment protocols, with only 88 transplants (76.5% of the total cohort) utilising both T cell depletion and RIC in the treatment protocol. This filtered cohort was analysed separately on the basis of the growing evidence that the elements of a clinical protocol has significant influence upon the outcome of KIR assessment models in transplantation, particularly in the context of interactions between NK cells and T cells.

In summary, this study identified that using donors with KIR B/x diplotypes in HPCT resulted in increased risk of GvHD grade II-IV and increase incidence of NRM, and receptor/ligand mismatched transplants may reduce incidence of NRM, evidenced in a cohort of T cell depleted RIC transplants (Cohort B). No model investigated in the presented cohort demonstrated any influence upon overall survival, event-free survival, or relapse.

Chapter 4 References

Boyington, J. C., Motyka, S. A., Schuck, P., Brooks, A. G. & Sun, P. D. (2000) Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. *Nature,* 405(6786), 537-43.

Bultitude, W. P., Schellekens, J., Szydlo, R. M., Anthias, C., Cooley, S. A., Miller, J. S., Weisdorf, D. J., Shaw, B. E., Roberts, C. H., Garcia-Sepulveda, C. A., Lee, J., Pearce, R. M., Wilson, M. C., Potter, M. N., Byrne, J. L., Russell, N. H., MacKinnon, S., Bloor, A. J., Patel, A., McQuaker, I. G., Malladi, R., Tholouli, E., Orchard, K., Potter, V. T., Madrigal, J. A., Mayor, N. P. & Marsh, S. G. E. (2020) Presence of donor-encoded centromeric KIR B content increases the risk of infectious mortality in recipients of myeloablative, T-cell deplete, HLA-matched HCT to treat AML. *Bone Marrow Transplant.*

Ciccone, E., Pende, D., Nanni, L., Di Donato, C., Viale, O., Beretta, A., Vitale, M., Sivori, S., Moretta, A. & Moretta, L. (1995) General role of HLA class I molecules in the protection of target cells from lysis by natural killer cells: evidence that the free heavy chains of class I molecules are not sufficient to mediate the protective effect. *Int Immunol,* 7(3), 393-400.

Cooley, S., Trachtenberg, E., Bergemann, T. L., Saeteurn, K., Klein, J., Le, C. T., Marsh, S. G., Guethlein, L. A., Parham, P., Miller, J. S. & Weisdorf, D. J. (2009) Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood,* 113(3), 726-32.

Cooley, S., Weisdorf, D. J., Guethlein, L. A., Klein, J. P., Wang, T., Le, C. T., Marsh, S. G., Geraghty, D., Spellman, S., Haagenson, M. D., Ladner, M., Trachtenberg, E., Parham, P. & Miller, J. S. (2010) Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood,* 116(14), 2411-9.

Cooley, S., Weisdorf, D. J., Guethlein, L. A., Klein, J. P., Wang, T., Marsh, S. G., Spellman, S., Haagenson, M. D., Saeturn, K., Ladner, M., Trachtenberg, E., Parham, P. & Miller, J. S. (2014) Donor killer cell Ig-like receptor B haplotypes, recipient HLA-C1, and HLA-C mismatch enhance the clinical benefit of unrelated transplantation for acute myelogenous leukemia. *J Immunol,* 192(10), 4592-600.

Davies, S. M., Iannone, R., Alonzo, T. A., Wang, Y. C., Gerbing, R., Soni, S., Kolb, E. A., Meshinchi, S., Orchard, P. J., Burns, L. J., Shenoy, S. & Leung, W. (2020) A Phase 2 Trial of KIR-Mismatched Unrelated Donor Transplantation Using in Vivo T Cell Depletion with Antithymocyte Globulin in Acute Myelogenous Leukemia: Children's Oncology Group AAML05P1 Study. *Biol Blood Marrow Transplant,* 26(4), 712-717.

Dehn, J., Spellman, S., Hurley, C. K., Shaw, B. E., Barker, J. N., Burns, L. J., Confer, D. L., Eapen, M., Fernandez-Vina, M., Hartzman, R., Maiers, M., Marino, S. R., Mueller, C., Perales, M. A., Rajalingam, R. & Pidala, J. (2019) Selection of unrelated donors and cord blood units for hematopoietic cell transplantation: guidelines from the NMDP/CIBMTR. *Blood,* 134(12), 924-934.

Gaetano, J. (2018) *Holm-Bonferroni sequential correction: An Excel calculator (1.3) [Microsoft Excel Workbook]* [Online]. Available: https://www.researchgate.net/publication/322568540_Holm-Bonferroni_sequential_correction_An_Excel_calculator_13 [Accessed].

Gentle, N. L., Loubser, S., Paximadis, M., Puren, A. & Tiemessen, C. T. (2017) Killer-cell immunoglobulin-like receptor (KIR) and human leukocyte antigen (HLA) class I genetic diversity in four South African populations. *Hum Immunol,* 78(7-8), 503-509.

Gonzalez-Galarza, F. F., Takeshita, L. Y., Santos, E. J., Kempson, F., Maia, M. H., da Silva, A. L., Teles e Silva, A. L., Ghattaoraya, G. S., Alfirevic, A., Jones, A. R. & Middleton, D. (2015) Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. *Nucleic Acids Res,* 43(Database issue), D784-8.

Hansasuta, P., Dong, T., Thananchai, H., Weekes, M., Willberg, C., Aldemir, H., Rowland-Jones, S. & Braud, V. M. (2004) Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. *Eur J Immunol,* 34(6), 1673-9.

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(3), 360-70.

Kärre, K., Ljunggren, H. G., Piontek, G. & Kiessling, R. (1986) Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature,* 319(6055), 675-8.

Krieger, E., Sabo, R., Moezzi, S., Cain, C., Roberts, C., Kimball, P., Chesney, A., McCarty, J., Keating, A., Romee, R., Wiedl, C., Qayyum, R. & Toor, A. (2020) Killer Immunoglobulin-Like Receptor-Ligand Interactions Predict Clinical Outcomes following Unrelated Donor Transplantations. *Biol Blood Marrow Transplant,* 26(4), 672-682.

Leung, W., Iyengar, R., Turner, V., Lang, P., Bader, P., Conn, P., Niethammer, D. & Handgretinger, R. (2004) Determinants of antileukemia effects of allogeneic NK cells. *The Journal of Immunology,* 172(1), 644-50.

Little, A. M., Green, A., Harvey, J., Hemmatpour, S., Latham, K., Marsh, S. G., Poulton, K. & Sage, D. (2016) BSHI Guidelines: HLA matching and donor selection for haematopoietic progenitor cell transplantation. *Int J Immunogenet,* 43(5), 263-86.

Machado-Sulbaran, A. C., Munoz-Valle, J. F., Ramirez-Duenas, M. G., Banos-Hernandez, C. J., Graciano-Machuca, O., Velarde-De la Cruz, E. E., Parra-Rojas, I. & Sanchez-Hernandez, P. E. (2017) Distribution of KIR genes and KIR2DS4 gene variants in two Mexican Mestizo populations. *Hum Immunol,* 78(10), 614-620.

Mandelboim, O., Reyburn, H. T., Valés-Gómez, M., Pazmany, L., Colonna, M., Borsellino, G. & Strominger, J. L. (1996) Protection from lysis by Natural Killer cells of group 1 and 2 specificity is mediated by residue 80 in human Histocompatibility Leukocyte Antigen C alleles and also occurs with empty Major Histocompatibility Complex molecules. *J Exp Med,* 184, 913-22.

Mayor, N. P., Hayhurst, J. D., Turner, T. R., Szydlo, R. M., Shaw, B. E., Bultitude, W. P., Sayno, J. R., Tavarozzi, F., Latham, K., Anthias, C., Robinson, J., Braund, H., Danby, R., Perry, J., Wilson, M. C., Bloor, A. J., McQuaker, I. G., MacKinnon, S., Marks, D. I., Pagliuca, A., Potter, M. N., Potter, V. T., Russell, N. H., Thomson, K. J., Madrigal, J. A. & Marsh, S. G. E. (2019) Recipients Receiving Better HLA-Matched Hematopoietic Cell Transplantation Grafts,

Uncovered by a Novel HLA Typing Method, Have Superior Survival: A Retrospective Study. *Biol Blood Marrow Transplant,* 25(3), 443-450.

Moretta, A., Sivori, S., Vitale, M., Pende, D., Morelli, L., Augugliaro, R., Bottino, C. & Moretta, L. (1995) Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. *J Exp Med,* 182(3), 875-84.

Murphy, W. J., Bennett, M., Kumar, V. & Longo, D. L. (1992) Donor-type activated natural killer cells promote marrow engraftment and B cell development during allogeneic bone marrow transplantation. *J Immunol,* 148(9), 2953-60.

Murphy, W. J., Kumar, V. & Bennett, M. (1987) Rejection of bone marrow allografts by mice with severe combined immune deficiency (SCID). Evidence that natural killer cells can mediate the specificity of marrow graft rejection. *J Exp Med,* 165(4), 1212-7.

Murphy, W. J., Kumar, V. & Bennett, M. (1990) Natural killer cells activated with interleukin 2 in vitro can be adoptively transferred and mediate hematopoietic histocompatibility-1 antigen-specific bone marrow rejection in vivo. *Eur J Immunol,* 20(8), 1729-34.

Nowak, J., Koscinska, K., Mika-Witkowska, R., Rogatko-Koros, M., Mizia, S., Jaskula, E., Polak, M., Mordak-Domagala, M., Lange, J., Gronkowska, A., Jedrzejczak, W. W., Kyrcz-Krzemien, S., Markiewicz, M., Dzierzak-Mietla, M., Tomaszewska, A., Nasilowska-Adamska, B., Szczepinski, A., Halaburda, K., Hellmann, A., Czyz, A., Gil, L., Komarnicki, M., Wachowiak, J., Baranska, M., Kowalczyk, J., Drabko, K., Gozdzik, J., Wysoczanska, B., Bogunia-Kubik, K., Graczyk-Pol, E., Witkowska, A., Marosz-Rudnicka, A., Nestorowicz, K., Dziopa, J., Szlendak, U., Warzocha, K., Lange, A. & Polish Donor-Recipient Matching Study, G. (2015) Role of donor activating KIR-HLA ligand-mediated NK cell education status in control of malignancy in hematopoietic cell transplant recipients. *Biol Blood Marrow Transplant,* 21(5), 829-39.

Nowak, J., Koscinska, K., Mika-Witkowska, R., Rogatko-Koros, M., Mizia, S., Jaskula, E., Polak, M., Mordak-Domagala, M., Lange, J., Gronkowska, A., Jedrzejczak, W. W., Kyrcz-Krzemien, S., Markiewicz, M., Dzierzak-Mietla, M., Tomaszewska, A., Nasilowska-Adamska, B., Szczepinski, A., Halaburda, K., Hellmann, A., Komarnicki, M., Gil, L., Czyz, A., Wachowiak, J., Baranska, M., Kowalczyk, J., Drabko, K., Gozdzik, J., Wysoczanska, B., Bogunia-Kubik, K., Graczyk-Pol, E., Witkowska, A., Marosz-Rudnicka, A., Nestorowicz, K., Dziopa, J., Szlendak, U., Warzocha, K., Lange, A. A. & Polish Donor-Recipient Matching Study, G. (2014) Donor NK cell licensing in control of malignancy in hematopoietic stem cell transplant recipients. *Am J Hematol,* 89(10), E176-83.

Petersdorf, E. W., Anasetti, C., Martin, P. J., Gooley, T., Radich, J., Malkki, M., Woolfrey, A., Smith, A., Mickelson, E. & Hansen, J. A. (2004) Limits of HLA mismatching in unrelated hematopoietic cell transplantation. *Blood,* 104(9), 2976-80.

Pical-Izard, C., Crocchiolo, R., Granjeaud, S., Kochbati, E., Just-Landi, S., Chabannon, C., Frassati, C., Picard, C., Blaise, D., Olive, D. & Fauriat, C. (2015) Reconstitution of natural killer cells in HLA-matched HSCT after reduced-intensity conditioning: impact on clinical outcome. *Biol Blood Marrow Transplant,* 21(3), 429-39.

Robinson, J., Barker, D. J., Georgiou, X., Cooper, M. A., Flicek, P. & Marsh, S. G. E. (2020) IPD-IMGT/HLA Database. *Nucleic Acids Res,* 48(D1), D948-D955.

Rolstad, B. & Benestad, H. B. (1991) Spontaneous alloreactivity of natural killer (NK) and lymphokine-activated killer (LAK) cells from athymic rats against normal haemic cells. NK cells stimulate syngeneic but inhibit allogeneic haemopoiesis. *Immunology,* 74(1), 86-93.

Ruggeri, L., Capanni, M., Casucci, M., Volpi, I., Tosti, A., Perruccio, K., Urbani, E., Negrin, R. S., Martelli, M. F. & Velardi, A. (1999) Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood,* 94(1), 333-9.

Saunders, P. M., Vivian, J. P., Baschuk, N., Beddoe, T., Widjaja, J., O'Connor, G. M., Hitchen, C., Pymm, P., Andrews, D. M., Gras, S., McVicar, D. W., Rossjohn, J. & Brooks, A. G. (2015) The interaction of KIR3DL1*001 with HLA class I molecules is dependent upon molecular microarchitecture within the Bw4 epitope. *J Immunol,* 194(2), 781-9.

Schetelig, J., Baldauf, H., Heidenreich, F., Massalski, C., Frank, S., Sauter, J., Stelljes, M., Ayuk, F. A., Bethge, W. A., Bug, G., Klein, S., Wendler, S., Lange, V., de Wreede, L. C., Furst, D., Kobbe, G., Ottinger, H. D., Beelen, D. W., Mytilineos, J., Fleischhauer, K., Schmidt, A. H. & Bornhauser, M. (2020) External validation of models for KIR2DS1/KIR3DL1-informed selection of hematopoietic cell donors fails. *Blood,* 135(16), 1386-1395.

Schmidt, A. H., Sauter, J., Baier, D. M., Daiss, J., Keller, A., Klussmeier, A., Mengling, T., Rall, G., Riethmuller, T., Schofl, G., Solloch, U. V., Torosian, T., Means, D., Kelly, H., Jagannathan, L., Paul, P., Giani, A. S., Hildebrand, S., Schumacher, S., Markert, J., Fussel, M., Hofmann, J. A., Schafer, T., Pingel, J., Lange, V. & Schetelig, J. (2020) Immunogenetics in stem cell donor registry work: The DKMS example (Part 2). *Int J Immunogenet,* 47(2), 139-148.

Shaw, B. E., Logan, B. R., Spellman, S. R., Marsh, S. G. E., Robinson, J., Pidala, J., Hurley, C., Barker, J., Maiers, M., Dehn, J., Wang, H., Haagenson, M., Porter, D., Petersdorf, E. W., Woolfrey, A., Horowitz, M. M., Verneris, M., Hsu, K. C., Fleischhauer, K. & Lee, S. J. (2018) Development of an Unrelated Donor Selection Score Predictive of Survival after HCT: Donor Age Matters Most. *Biol Blood Marrow Transplant,* 24(5), 1049-1056.

Shaw, B. E., Mayor, N. P., Szydlo, R. M., Bultitude, W. P., Anthias, C., Kirkland, K., Perry, J., Clark, A., Mackinnon, S., Marks, D. I., Pagliuca, A., Potter, M. N., Russell, N. H., Thomson, K., Madrigal, J. A. & Marsh, S. G. E. (2017) Recipient/donor HLA and CMV matching in recipients of T-cell-depleted unrelated donor haematopoietic cell transplants. *Bone Marrow Transplant,* 52(5), 717-725.

Shimoni, A., Labopin, M., Lorentino, F., Van Lint, M. T., Koc, Y., Gulbas, Z., Tischer, J., Bruno, B., Blaise, D., Pioltelli, P., Afanasyev, B., Ciceri, F., Mohty, M. & Nagler, A. (2019) Killer cell immunoglobulin-like receptor ligand mismatching and outcome after haploidentical transplantation with post-transplant cyclophosphamide. *Leukemia,* 33(1), 230-239.

Vierra-Green, C., Roe, D., Jayaraman, J., Trowsdale, J., Traherne, J., Kuang, R., Spellman, S. & Maiers, M. (2016) Estimating KIR haplotype frequencies on a cohort of 10,000 individuals: A comprehensive study on population variations, typing resolutions, and reference haplotypes. *PLoS One,* 11(10), e0163973.

Weisdorf, D., Cooley, S., Wang, T., Trachtenberg, E., Haagenson, M. D., Vierra-Green, C., Spellman, S., Spahn, A., Vogel, J., Kobusingye, H., Fehninger, T., Woolfrey, A., Devine, S., Ross, M., Waller, E. K., Sobecks, R., Parham, P., Guethlein, L. A., Marsh, S. G. E., Miller, J. & participating center writing, c. (2019) KIR donor selection: Feasibility in identifying better donors. *Biol Blood Marrow Transplant,* 25(1), e28-32.

Willem, C., Makanga, D. R., Guillaume, T., Maniangou, B., Legrand, N., Gagne, K., Peterlin, P., Garnier, A., Bene, M. C., Cesbron, A., Le Bourgeois, A., Chevallier, P. & Retiere, C. (2019) Impact of KIR/HLA Incompatibilities on NK Cell Reconstitution and Clinical Outcome after T Cell-Replete Haploidentical Hematopoietic Stem Cell Transplantation with Posttransplant Cyclophosphamide. *J Immunol,* 202(7), 2141-2152.

Chapter 5 | The influence of killer-cell immunoglobulin-like receptor gene allele groups encoding C1 and C2 epitope-specific receptors upon T cell depleted reduced intensity conditioning haematopoietic progenitor cell transplantation outcomes

Summary

A majority of established KIR assessment algorithms in donor selection for haematopoietic progenitor cell transplantation (HPCT) use KIR gene content (presence/absence) of the KIR gene complex, but comparatively little is known about the implications of KIR allelic polymorphism in this setting. This study investigated the influence of allelic polymorphism in the inhibitory KIR genes encoding receptors specific for the C1 and C2 epitopes (KIR2DL2/3 and KIR2DL1 respectively), assessing the donor KIR genotype against post-HPCT outcomes. KIR2DL1 and KIR2DL2/3 allele groups displayed close linkage, due to the proximity of all three genes within the centromeric region of the KIR gene complex, negating the value of independently assessing allelic polymorphism in all three genes. KIR2DL1 allele groups in the donor significantly impacted upon 3-year HPCT outcomes in T cell depleted reduced intensity conditioning (RIC) transplantation. Donors possessing KIR2DL1*001/002 and/or 004 allele groups were found to significantly improve post-transplant outcomes. KIR2DL1*001/002 allele group positive donors improved 3-year overall survival (OS) (61.5% vs 34.0%, p=0.017, p_c=0.085) and event-free survival (EFS) (53.9% vs. 29.8%, p=0.032, p_c=0.096). KIR2DL1*004 allele group positive donors trended towards improving 3-year OS (62.2% vs 40.3%, p=0.066, p_c=0.184) and EFS (54.2% vs 35.5%, p=0.077, p_c=0.154), and also significantly reduced relapse incidence (22.9% vs 48.4%, p=0.046, p_c=0.184). KIR2DL1*003 allele group positive donors were detrimental to post-transplant outcomes, reducing 3-year OS (36.5% vs 61.8%, p=0.046, p_c=0.184) and EFS (28.9% vs 58.8%, p=0.015, p_c=0.084), and increasing 3-year relapse incidence (54.1% vs 21.9%, p=0.019, p_c=0.090). The data suggests donor selection algorithms for T cell depleted RIC HPCT should consider avoiding selection of KIR2DL1*003 allele group positive donors, where possible, and contributes to the mounting evidence that KIR assessment in donor selection algorithms should be assessed in the context of the conditioning regime protocol used.

5.1 Introduction

KIR allelic polymorphism dramatically alters the characteristics of the transcribed glycoprotein, influencing the binding avidity, surface expression, and signalling capacity of the expressed receptor (Boudreau et al., 2014; Forlenza et al., 2016). The precise phenotypical alteration is principally dependent upon where within the gene the polymorphism is located, with exons 3 to 5 (D0-D2 extracellular regions) associated with binding avidity, and exons 7 to 9 (transmembrane and cytoplasmic regions) associated with signalling capacity (Mulrooney et al., 2008; Bari et al., 2009; Sharma et al., 2009). Mutations within any part of the gene could potentially affect surface expression (Uhrberg, 2005; VandenBussche et al., 2006; Alicata et al., 2016; Le Luduec et al., 2019).

KIR2DL1, 2DL2 and 2DL3 represent some of the most prominent inhibitory KIR receptors in a NK cell's repertoire. These receptors bind amino acid residue 80 of the HLA-Cw protein complex, with KIR2DL1 primarily displaying avidity for lysine at this position (C2 epitope) and KIR2DL2 and 2DL3 primarily binding asparagine (C1 epitope) (Colonna et al., 1992; Mandelboim et al., 1996; Winter et al., 1998; Moesta et al., 2008). These receptor/ligand interactions are essential for the NK cell immune response, with a consequent detrimental impact upon post-transplant outcomes for acute myelogenous leukaemia (AML) if a C1 ligand is absent (Neuchel et al., 2017; Shimoni et al., 2017). Conversely, absence of the C2 ligand presents an improvement in post-transplant outcomes, with reduced incidence of relapse (Arima et al., 2018).

In recent years, there has been a concerted effort to investigate the relationship between KIR genomics and proteomics, to better understand how allelic polymorphism can be used to predict the activity of receptor glycoproteins.

Phylogenetic research has established defined allele groups in a number of KIR genes, based upon sequence homology within each allele group (Boudreau et al., 2014; Hilton et al., 2015a). In KIR3DL1, these allele groups have been shown to approximately correlate with phenotypical characteristics of the respective gene product, with alleles within each phylogenetic clade consistently presenting similar expression levels, with the exception of the low surface density KIR3DL1*007 allele group contained within the high surface density KIR3DL1*002 allele group (Boudreau et al., 2014). Binding avidity can also be dependent upon the peptide presented by the HLA as part of antigen presentation (Hansasuta et al., 2004; O'Connor et al., 2015; Sim et al., 2017).

Polymorphism within KIR2DL1 has previously been demonstrated to impact post-transplant outcomes. Donors possessing KIR2DL1 alleles with arginine at residue 245 (KIR2DL1-R^{245}) present improvements in overall survival (OS) and progression-free survival in paediatric patients compared to donors who only possess cysteine at this position (KIR2DL1-C^{245}) (Bari et al., 2013). Position 245 of KIR2DL1 is located within the transmembrane region, with KIR2DL1-R^{245} receptors exhibiting increased signalling capacity compared to KIR2DL1-C^{245} due to increased recruitment of β-Arrestin 2, which in turn increases the recruitment of SHP-2 tyrosine phosphatase (Bari et al., 2009).

Recent advancements in next generation sequencing (NGS) has facilitated routine genetic sequencing of KIR genes in clinical histocompatibility & immunogenetics laboratories, but the influence of allelic polymorphism of KIR receptors specific for C1 and C2 epitopes (KIR2DL2/3 and KIR2DL1 respectively) remains relatively unexplored when compared to the research into KIR3DL1 and its ligand, HLA-Bw4.

The aim of this study was to expand upon existing research into the influence of KIR2DL1, 2DL2, and 2DL3 allelic polymorphism upon HPCT by investigating the effects of the respective allele groups upon transplant outcomes.

5.2 Methods

5.2.1 Cohort Selection

The inclusion and exclusion criteria was identical to that described in Chapter 4.2.1.

One further transplant pair was excluded from the study due to failure of KIR gene sequencing for the respective donor. One donor failed sequencing at KIR2DL1, and so was only assessed at KIR2DL2/3.

The final cohort sizes were as follows: Cohort A (KIR2DL1 n=113, KIR2DL2/3 n=114), and Cohort B (KIR2DL1 n=86, KIR2DL2/3 n=87).

5.2.2 Allele Group Definition

Each donor KIR2DL1, 2DL2, and 2DL3 genotype was categorised into allele groups. Allele groups were defined in a multi-step process. All KIR allele sequences defined in the cohort were aligned, and positions of polymorphisms recorded. Any 'private' SNPs present in only a single allele were excluded. The 'public' SNPs common among multiple alleles, were then translated into their amino acid assignments, facilitating the exclusion of synonymous mutations. The alleles were then grouped according to amino acid sequence profiles. These amino acid sequence profiles were compared against published KIR phylogenetic clades data, identifying classification of allele groups in accordance with phylogenetic clades (Hilton et al., 2015a). A minimal number of amino acid residue motifs were chosen to define an allele group within the cohort: two positions for KIR2DL1, one position for KIR2DL3. All

KIR2DL2 alleles identified within the donor cohort were within the same phylogenetic clade (the KIR2DL2*001 allele group), and so no additional classification on the basis of signature residue motifs was required. Allele group definitions used in the study are described in Table 25.

5.2.3 Statistical Analysis

Frequencies for each characteristic were calculated according to proportional representation of each respective classification within the total sample size. All Kaplan Meier survival curves and HR were calculated using IBM SPSS Statistics v25. Kaplan Meier survival curves were constructed for primary and secondary transplant outcomes factors, with log rank regression and cox regression (with associated hazard ratio data) also calculated. Statistical significance was considered to be $p \leq 0.05$. Holm-Bonferonni sequential correction was carried out for all statistics to provide a corrected p value (p_c) (Gaetano, 2018). The p_c values are only presented where the respective $p<0.10$.

5.2.4 Transplant Outcome Definitions

Definitions are described in Chapter 4.2.4.

5.2.5 Ethics

Ethical approval is described in Chapter 4.2.5.

5.3 Results

5.3.1 Transplant Characteristics

Transplant demographic data are described in Chapter 4.3.1 (demographic data did not differ significantly between the selected cohorts of each Chapter).

The KIR allele group demographic data is presented in Table 25. The KIR2DL1*003 group was the most common KIR2DL1 allele group within the donor cohort, with 60.5% of donors possessing this characteristic, followed by KIR2DL1*001/002 group (45.3%), and KIR2DL1*004 group (27.9%). Of the KIR2DL3 alleles, KIR2DL3*001 group (63.2%) was more common than the KIR2DL3*002 group (44.8%).

Allele Group	KIR alleles identified within cohort (KIR2DL1: n=113, KIR2DL2/3: n=114)	Defining polymorphism	Number donors possessing allele group in Cohort A (%) (KIR2DL1: n=113, KIR2DL2/3: n=114)	Number donors possessing allele group in Cohort B (%) (KIR2DL1: n=86, KIR2DL2/3: n=87)
KIR2DL1*001/002 group	KIR2DL1* 001, 002, 008	KIR2DL1 Positions: P^{114}, R^{245}	53 (46.9%)	39 (45.3%)
KIR2DL1*003 group	KIR2DL1* 003, 020, 032N, 034	KIR2DL1 Positions: L^{114}, R^{245}	68 (60.2%)	52 (60.5%)
KIR2DL1*004 group	KIR2DL1* 004, 007, 011	KIR2DL1 Positions: P^{114}, C^{245}	29 (25.7%)	24 (27.9%)
KIR2DL3*001 group	KIR2DL3* 001, 003, 013	KIR2DL3 Position: R^{297}	71 (62.3%)	55 (63.2%)
KIR2DL3*002 group	KIR2DL3* 002, 005, 006, 012, 015	KIR2DL3 Position: H^{297}	53 (46.5%)	39 (44.8%)
KIR2DL2*001 group	KIR2DL2* 001, 003, 006	KIR2DL2 positive (no KIR2DL2*004 group present within cohort)	62 (54.4%)	49 (56.3%)

Table 25 – KIR allele group definitions for KIR2DL1 and KIR2DL2/3, and frequencies of each allele group within Cohort A (n=114) and Cohort B (n=87).

KIR allele groups were defined in accordance with the allele groups identified by Hilton et al. (2015a). One donor sample positive for KIR2DL1 failed sequencing for allelic assignment, and so was excluded from analysis for alleles of this gene.

5.3.2 KIR2DL1 and KIR2DL2/3 allele groups exhibit associations both with KIR diplotype profiles and between KIR allele groups

The KIR allele group frequencies in donors of Cohort A (where the KIR diplotype profile was identified in ≥5 donors) are displayed in Table 26. A high degree of association between KIR allele groups and KIR diplotype profiles was present, with all KIR haplotype profiles observed displaying restriction to between one and four

different KIR2DL1 and KIR2DL2/3 allele group combinations from the many potential permutations.

Only two KIR diplotype profiles negative for KIR2DL1 were identified within the investigated donor pool: KIR B/x72 (5 donors) and B/x76 (1 donor). All six of these donors only possessed KIR2DL2 alleles of the KIR2DL2*001 group (KIR2DL2*00101 and/or 00301), and were negative for KIR2DL3.

		KIR2DL3*001 grp (n=54)	KIR2DL3*002 grp (n=39)	KIR2DL2*001 grp (n=49)
KIR2DL1*001/002 grp (n=39)	PPV	17.0%	97.9%	31.9%
	NPV	38.5%	97.4%	43.6%
	Pearson Chi Sq.	18.08 (p=<0.001, p_c=<0.001)	78.12 (p=<0.001, p_c=<0.001)	5.22 (p=0.022, p_c=0.044)
KIR2DL1*003 grp (n=52)	PPV	94.1%	23.5%	23.5%
	NPV	100%	25.1%	44.2%
	Pearson Chi Sq.	77.94 (p=<0.001, p_c=<0.001)	21.98 (p=<0.001, p_c=<0.001)	8.72 (p=0.003, p_c=0.012)
KIR2DL1*004 grp (n=24)	PPV	27.4%	58.1%	58.1%
	NPV	37.5%	54.2%	95.8%
	Pearson Chi Sq.	9.11 (p=0.003, p_c=0.012)	1.04 (p=0.307, p_c=0.307)	20.50 (p=<0.001, p_c=<0.001)

Figure 27 – Heat map displaying positive predictive values (PPV) and negative predictive values (NPV) of KIR2DL1 and KIR2DL2/3 allele group linkages among the donor cohort (Cohort A, n=113).
Colour intensity indicative of digression from 50% PPV/NPV: red = >50% PPV/NPV, blue = <50% PPV/NPV. One donor of the cohort was excluded from association analysis due to failed KIR2DL1 sequencing.

Tight linkage was also observed between KIR allele groups (Figure 27). Of the donors positive for the KIR2DL1*001/002 group, 98.1% (52 of 53) were also positive for the KIR2DL3*002 group (97.9% PPV), and 98.4% (60 of 61) of donors negative for the KIR2DL1*001/002 group were also negative for the KIR2DL2/3*002 group (97.4% NPV). A similar association was observed between the KIR2DL1*003 group and KIR2DL2/3*001 group, with 97.1% (68 of 70) of donors positive for KIR2DL1*003 group also positive for KIR2DL3*001 group (94.1% PPV), and 100% (43 of 43) donors negative for KIR2DL1*003 group were also negative for KIR2DL2/3*002 group (100% NPV).

KIR Diplotype Identity	KIR Allele Group Profile n (frequency in relation to all individuals carrying respective KIR diplotype profile)									
	KIR2DL1 Negative	KIR2DL1* 001 group	KIR2DL1* 001 group	KIR2DL1* 001 group	KIR2DL1* 003 group	KIR2DL1* 003 group	KIR2DL1* 004 group	KIR2DL1* 001+003 groups	KIR2DL1* 001+004 groups	KIR2DL1* 003+004 groups
	KIR2DL2*001 group	KIR2DL3*002 group	KIR2DL3* 001+002 groups	KIR2DL3* 002 group + KIR2DL2* 001 group	KIR2DL3*001 group	KIR2DL3* 001 group + KIR2DL2* 001 group	KIR2DL3* 001 group + KIR2DL2* 001 group	KIR2DL3* 001+002 groups	KIR2DL3* 002 group + KIR2DL2* 001 group	KIR2DL3* 001 group + KIR2DL2* 001 group
A/A1	-	9 (26.5%)	1 (2.9%)	-	12 (35.3%)	-	-	12 (35.3%)	-	-
B/x2	-	3 (25.0%)	-	-	4 (33.3%)	-	-	5 (38.4%)	-	-
B/x3	-	-	-	2 (33.3%)	-	4 (66.7%)	-	-	-	-
B/x4	-	-	-	6 (28.6%)	-	15 (71.4%)	-	-	-	-
B/x5	-	-	-	-	-	-	-	-	5 (50.0%)	5 (50.0%)
B/x6	-	-	-	-	-	-	1 (11.1%)	-	5 (88.9%)	3 (33.3%)
B/x72	5 (100%)	-	-	-	-	-	-	-	-	-

Table 26 – KIR allele group profile frequencies within Cohort A for KIR2DL1 and KIR2DL2/3 allele groups in relation to the respective individual's KIR diplotype profile.

Only KIR diplotype profiles with ≥5 individuals in the total donor cohort (n=114) are described, representing 85.1% (n=97) of the cohort. Frequencies are presented as percentage distribution within each KIR diplotype identity profile. A dash indicates a frequency of 0. KIR diplotype B/x72 is negative for KIR2DL1 by definition.

The weaker negative associations observed between the KIR2DL1*001/002 group and KIR2DL2/3*001 group was largely accounted for by allele group heterozygosity. Of the donors positive for both the KIR2DL1*001/002 group and KIR2DL3*001 group, 95.2% (20 of 21) were KIR2DL1*001/002, 003 group heterozygous. Likewise, 54.5% (6 of 11) of individuals negative for both these allele groups were negative for KIR2DL1, and the remaining 45.4% (5 of 11) were homozygous for KIR2DL1*004. These data suggest that KIR2DL1*001/002 group alleles and KIR2DL3*001 group alleles may not be commonly found on the same haplotype.

KIR2DL1*003 and KIR2DL3*002 groups also presented indications that these two groups are generally mutually exclusive, with KIR allele group heterozygosity also accounted for the weak association. All donors positive for both KIR2DL1*003 and KIR2DL3*002 groups were KIR2DL1*003 and 001/002 group heterozygous. The

composition of donors negative for both KIR2DL1*003 and KIR2DL3*002 groups were the same eleven donors previously described as donors negative for both KIR2DL1*001/002 and KIR3*001 groups.

The KIR2DL1*004 group and KIR2DL2*001 group did not present any strong association with other allele groups of the other investigated KIR genes, with a weak PPV of 58.1%. However, a strong NPV of 95.8% was observed, suggesting that although KIR2DL2*001 allele group is not restricted to association with the KIR2LD1*004 group, the KIR2DL1*004 group is highly restricted to association with the KIR2DL2*001 group.

Due to the close association described for KIR2DL1*001/002 group+KIR3DL2*002 group and KIR2DL1*003 group+KIR2DL3*001 group, these data provided similar findings. The findings of KIR2DL3*001 and 002 group donor transplant data will not be described, as these mirror the findings of their associated KIR2DL1 group donor transplants. Data for these groups is available in Appendix A.iii.

Hazard ratios for the relationship between KIR allele groups and transplant outcomes for Cohorts A and B can be found in Table 27.

All subsequent data describes Cohort B unless otherwise stated. Kaplan Meier curves for Cohort A can be found in Appendix A.iii.

KIR Gene Group in Transplant Donor	Cohort	Category	n (%)	All-cause Mortality HR (95% CI)	Non-Relapse Mortality HR (95% CI)	Event HR (95% CI)	Relapse HR (95% CI)	GvHD grade II-IV HR (95% CI)
KIR2DL1* 001 group	Cohort A (All Transplants)	Negative	60 (53.1%)	1	1	1	1	1
		Positive	53 (46.9%)	0.66 (0.39-1.12) (p=0.123)	1.31 (0.52-3.33) (p=0.566)	0.76 (0.47-1.26) (p=0.287)	0.73 (0.38-1.41) (p=0.346)	0.67 (0.36-1.25) (p=0.207)
	Cohort B (T cell Depleted RIC Transplants)	Negative	47 (54.7%)	1	1	1	1	1
		Positive	39 (45.3%)	0.48 (0.25-0.89) (p=0.020, p_c=0.080)	0.89 (0.30-2.66) (p=0.837)	0.54 (0.30-0.96) (p=0.035, p_c=0.105)	0.56 (0.27-1.17) (p=0.125)	0.71 (0.35-1.45) (p=0.351)
KIR2DL1* 003 group	Cohort A	Negative	45 (39.8%)	1	1	1	1	1
		Positive	68 (60.2%)	1.41 (0.81-2.44) (p=0.224)	0.70 (0.28-1.76) (p=0.443)	1.49 (0.88-2.51) (p=0.136)	1.69 (0.83-3.41) (p=0.147)	1.20 (0.64-2.23) (p=0.575)
	Cohort B	Negative	34 (39.5%)	1	1	1	1	1
		Positive	52 (60.5%)	1.91 (1.00-3.63) (p=0.050, p_c=0.150)	0.82 (0.28-2.44) (p=0.722)	2.12 (1.14-3.94) (p=0.017, p_c=0.068)	2.65 (1.14-6.17) (p=0.024, p_c=0.096)	0.99 (0.49-2.01) (p=0.978)
KIR2DL1* 004 group	Cohort A	Negative	84 (74.3%)	1	1	1	1	1
		Positive	29 (25.7%)	0.57 (0.30-1.10) (p=0.094, p_c=0.376)	1.20 (0.45-3.21) (p=0.712)	0.59 (0.32-1.09) (p=0.091, p_c=0.364)	0.45 (0.19-1.09) (p=0.077, p_c=0.308)	1.56 (0.83-2.93) (p=0.170)
	Cohort B	Negative	62 (72.1%)	1	1	1	1	1
		Positive	24 (27.9%)	0.51 (0.25-1.06) (p=0.072, p_c=0.150)	0.60 (0.44-4.15) (p=0.596)	0.55 (0.28-1.08) (p=0.082, p_c=0.164)	0.39 (0.15-1.02) (p=0.055, p_c=0.165)	1.72 (0.85-3.48) (p=0.134)
KIR2DL2* 001 group	Cohort A	Negative	52 (47.4%)	1	1	1	1	1
		Positive	62 (52.6%)	1.07 (0.64-1.81) (p=0.797)	1.67 (0.63-4.44) (p=0.307)	1.01 (0.62-1.65) (p=0.966)	0.88 (0.46-1.67) (p=0.684)	1.90 (1.00-3.61) (p=0.050, p_c=0.200)
	Cohort B	Negative	38 (46.0%)	1	1	1	1	1
		Positive	49 (54.0%)	1.16 (0.65-2.09) (p=0.619)	2.66 (0.73-9.66) (p=0.137)	1.16 (0.66-2.03) (p=0.600)	0.98 (0.48-1.99) (p=0.957)	2.01 (0.95-4.25) (p=0.067, p_c=0.268)

Table 27 – Hazard ratios (HR) of donor KIR2DL1 and KIR2DL2 allele groups in post-transplant outcomes in Cohort A (n=114) and B (n=88).

Key: Data with p≤0.05 are shaded blue. Data with 0.05<p≤0.10 are shaded orange.

5.3.3 Overall Survival

Donors positive for the KIR2DL1*001/002 group resulted in transplants with improved 3-year OS, and KIR2DL1*004 group donors trended towards a similar outcome (Figure 28). 61.5% versus 34.0% 3-year OS was observed in KIR2DL1*001/002 group positive and negative donor transplants respectively

(p=0.017, p_c=0.085). This translate to a hazard ratio of 0.48 (95% CI=0.25-0.89, p=0.020, p_c=0.080) for transplants using donors possessing the KIR2DL1*001/002 group compared to transplants using donors negative for this allele group. KIR2DL1*004 positive donor transplants trended towards improving OS, with 62.2% 3-year OS in KIR2DL1*004 positive donor transplants compared to 40.3% in KIR2DL1*004 negative donor transplants (p=0.066, p_c=0.184).

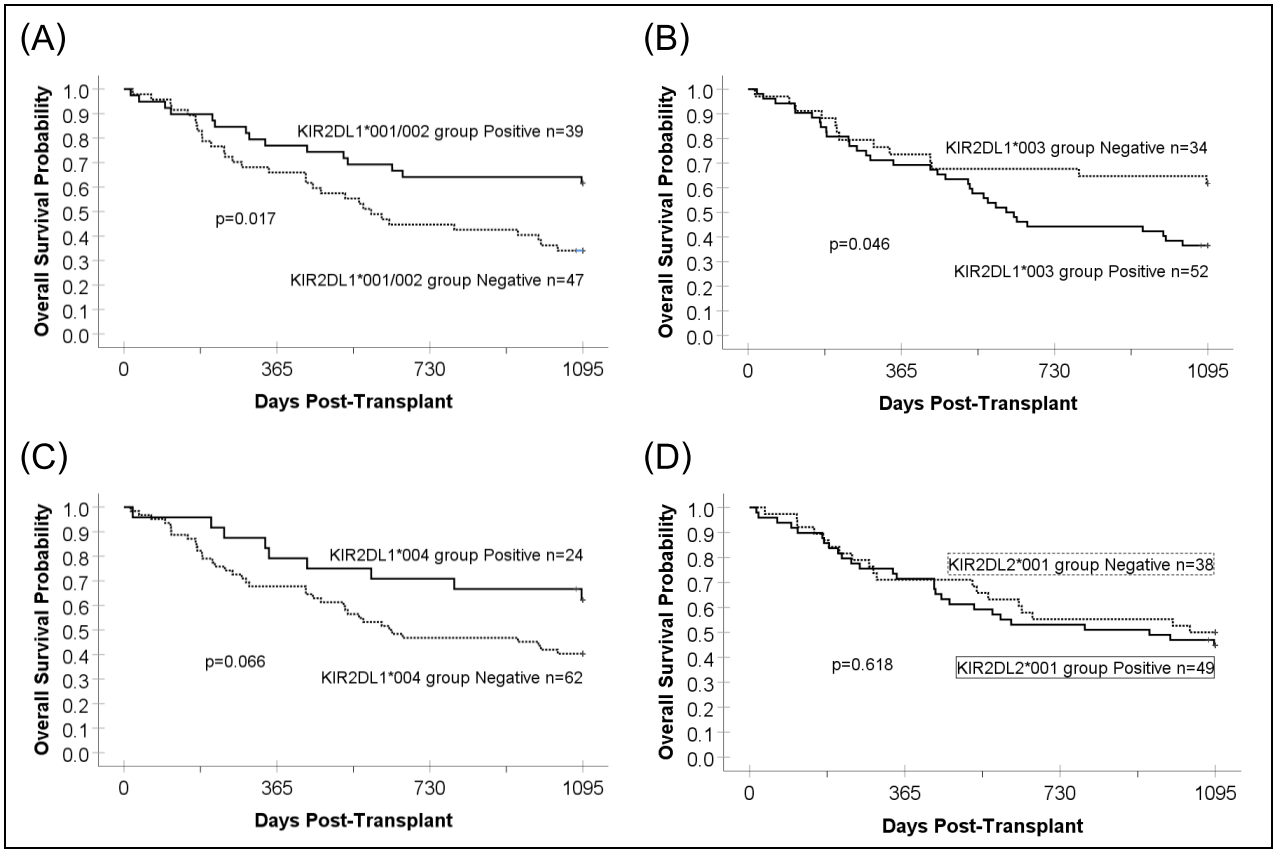


Figure 28 – The impact of donors possessing KIR2DL1 (n=86) and KIR2DL2 (n=87) allele group presence/absence upon 3-year post-transplant overall survival (OS) in T cell depleted RIC HPCT (Cohort B).

(A) KIR2DL1*001/002 group positive donors significantly improve OS (p=0.017, p_c=0.085). (B) KIR2DL1*003 group positive donors significantly reduce 3-year OS (p=0.046, p_c=0.184). (C) Transplants using KIR2DL1*004 allele group positive donors trend towards improving OS (p=0.066, p_c=0.184). (D) KIR2DL2*001 group presence/absence offers no influence upon 3-year OS (p=0.618).

In contrast, KIR2DL1*003 group positive donors provide inferior 3-year OS. 36.5% 3-year OS was observed in transplants using KIR2DL1*003 group positive donors,

compared to 61.8% in transplants KIR2DL1*003 group negative donors (p=0.046, p_c=0.184) (HR=1.91, 95% CI=1.00-3.63, p=0.050, p_c=0.150).

KIR2DL2*001 group donors did not influence OS. KIR2DL2*001 group positive donors and KIR2DL2*001 group negative donors resulted in 3-year OS of 44.8% and 50.0% respectively (p=0.618).

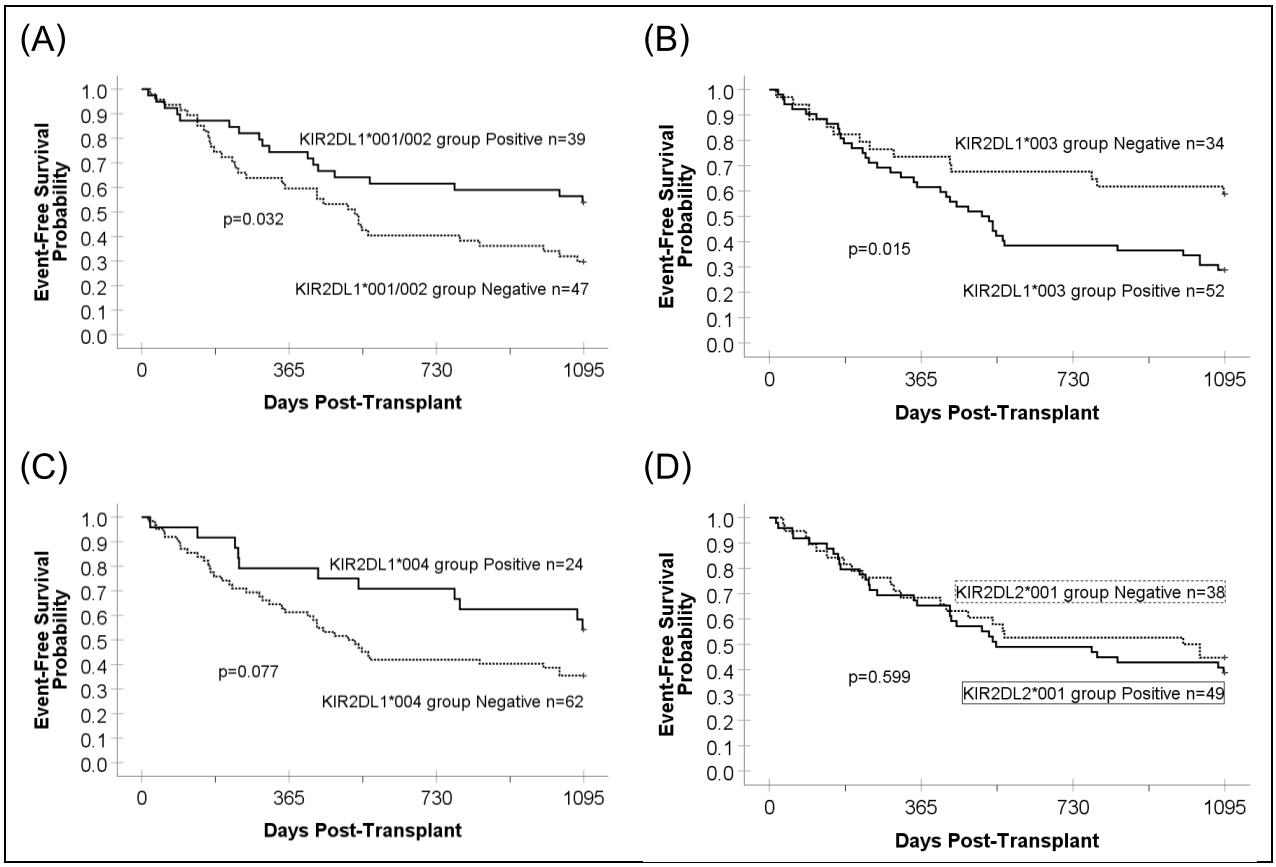


Figure 29 – The impact of donor KIR2DL1 (n=86) and KIR2DL2/3 (n=87) group presence/absence upon 3-year post-transplant event free survival (EFS) in T cell depleted RIC HPCT (Cohort B).

(A) Transplants using KIR2DL1*001/002 group positive donors experienced significantly improved EFS (p=0.032, p_c=0.096). (B) KIR2DL1*003 group positive donors significantly reduce EFS (p=0.015, p_c=0.084). (C) KIR2DL1*004 group positive donors trended towards improving EFS (p=0.077, p_c=0.154). (D) KIR2DL2*001 presence/absence in the donor did not influence 3-year post-transplant EFS (p=0.599).

In Cohort A, KIR2DL1 allele groups did not significantly impact upon 3-year OS. In KIR2DL1*001/002 group positive donor transplants, 57.4% 3-year OS was observed, compared to 41.4% in the KIR2DL1*001/002 group negative donor transplants (p=0.120). KIR2DL1*004 group positive donor transplants trended towards

improving 3-year OS, with 65.5% 3-year OS compared to 44.2% in KIR2DL1*004 group negative donor transplants (p=0.090, p_c=0.450).

5.3.4 Event-Free Survival

Investigation of EFS provided similar findings to those of OS, with KIR2DL1*001/002 group positive donors significantly improving EFS, and KIR2DL1*004 group positive donors trending towards comparably advantageous outcomes (Figure 29, Table 27). At 3 years, KIR2DL1*001/002 group positive donor transplants presented 53.9% EFS, compared to 29.8% in transplants using donors negative for this group (p=0.032, p_c=0.096). KIR2DL1*001/002 group positive donor transplants provided a HR of 0.54 (95% CI=0.30-0.96, p=0.035, p_c=0.105) compared to negative donor transplants. KIR2DL1*004 group positive donors presented a trend towards improving transplant outcomes. Transplants using KIR2DL1*004 group positive donors resulted in a 3-year EFS of 54.2% compared to 35.5% in KIR2DL1*004 group negative donor transplants (p=0.077, p_c=0.154).

KIR2DL1*003 group positive donors were detrimental to EFS. The 3-year EFS of KIR2DL1*003 group positive donors was 28.9%, in contrast to 58.8% in transplants using donors negative for this allele group (p=0.015, p_c=0.084). The HR of KIR2DL1*003 group positive donor transplants experiencing an event was 2.12 (95% CI=1.14-3.94, p=0.017, p_c=0.068) compared to KIR2DL1*003 group negative donor transplants.

KIR2DL1 and KIR2DL2/3 groups did not significantly influence EFS in Cohort A. KIR2DL1*004 group positive donors trended towards improving EFS, with a 54.7% 3-year EFS in KIR2DL1*004 group positive donor transplants compared to 38.8% in

negative donor transplants (p=0.087, p_c=0.522). All other findings for Cohort A did not reach significance in EFS.

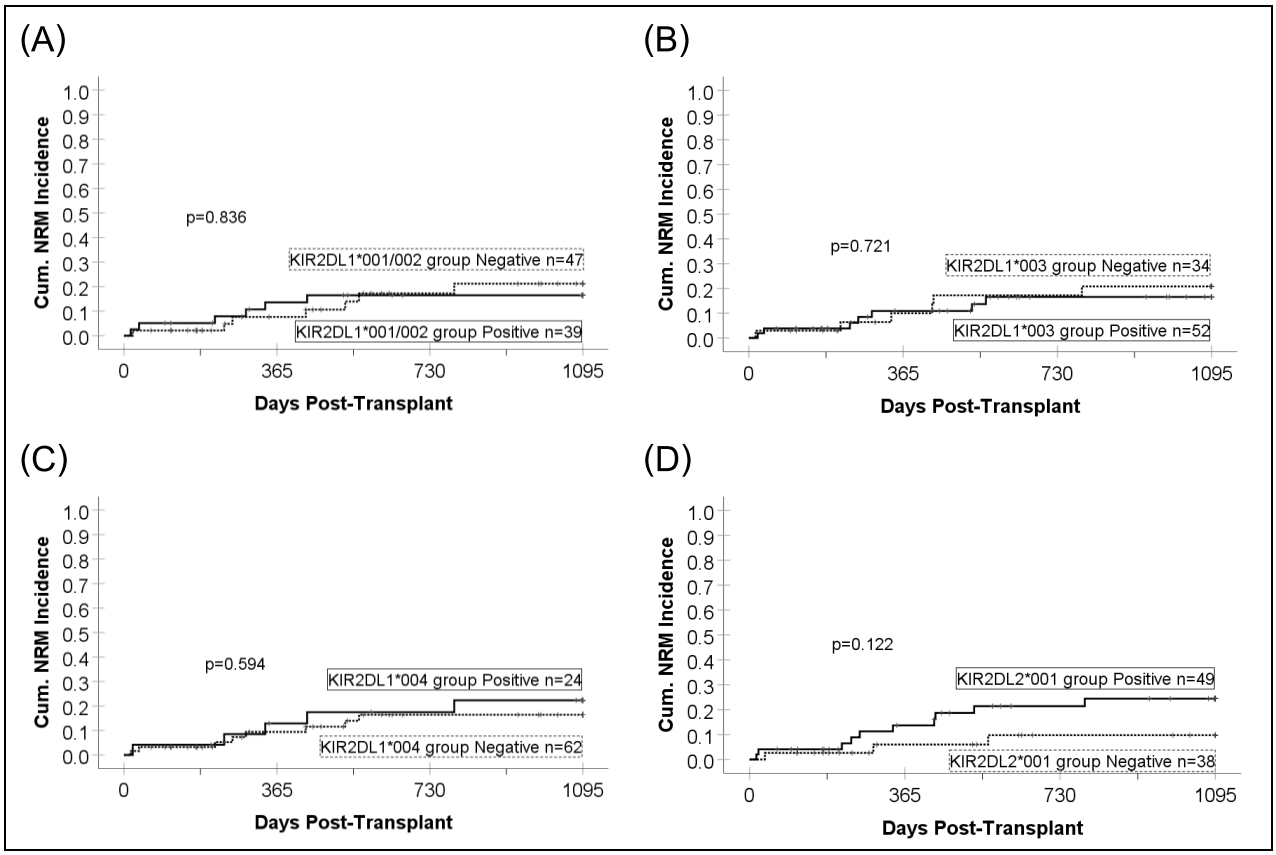


Figure 30 – The impact of donor KIR2DL1 (n=86) and KIR2DL2 (n=87) allele group presence/absence upon 3-year post-transplant non-relapse mortality (NRM) in T cell depleted RIC HPCT (Cohort B).

(A) Transplants using KIR2DL1*001/002 group positive/negative donors (p=0.836) (B) Transplants using KIR2DL1*003 group positive/negative donors. (p=0.721) (C) Transplants using KIR2DL1*004 group positive/negative donors (p=0.594) (D) Transplants using KIR2DL2*001 group positive/negative donors (p=0.122). No KIR2DL1/2 allele groups in donor significantly influenced 3-year post-transplant NRM.

5.3.5 Non-Relapse Mortality

KIR2DL1 and KIR2DL2 groups did not significantly influence NRM (Figure 30). KIR2DL2*001 group positive donors transplants presented separation of strata in the Kaplan Meier curve, but this did not reach significance (24.4% versus 9.8% 3-year NRM, p=0.122). Similar findings were observed in Cohort A.

5.3.6 Relapse

KIR2DL1*003 group presence/absence in the donor most significantly influenced relapse rates, with a large detrimental effect (Table 28, Figure 31). A 3-year relapse rate of 54.1% was observed in KIR2DL1*003 group positive donor transplants, in contrast to 21.9% in KIR2DL1*003 group negative donor transplants (p=0.019, p_c=0.090). KIR2DL1*003 group positive donor transplants offered a HR for experiencing relapse within 3 years post-transplant of 2.45 (95% CI=1.14-6.17, p=0.024, p_c=0.096).

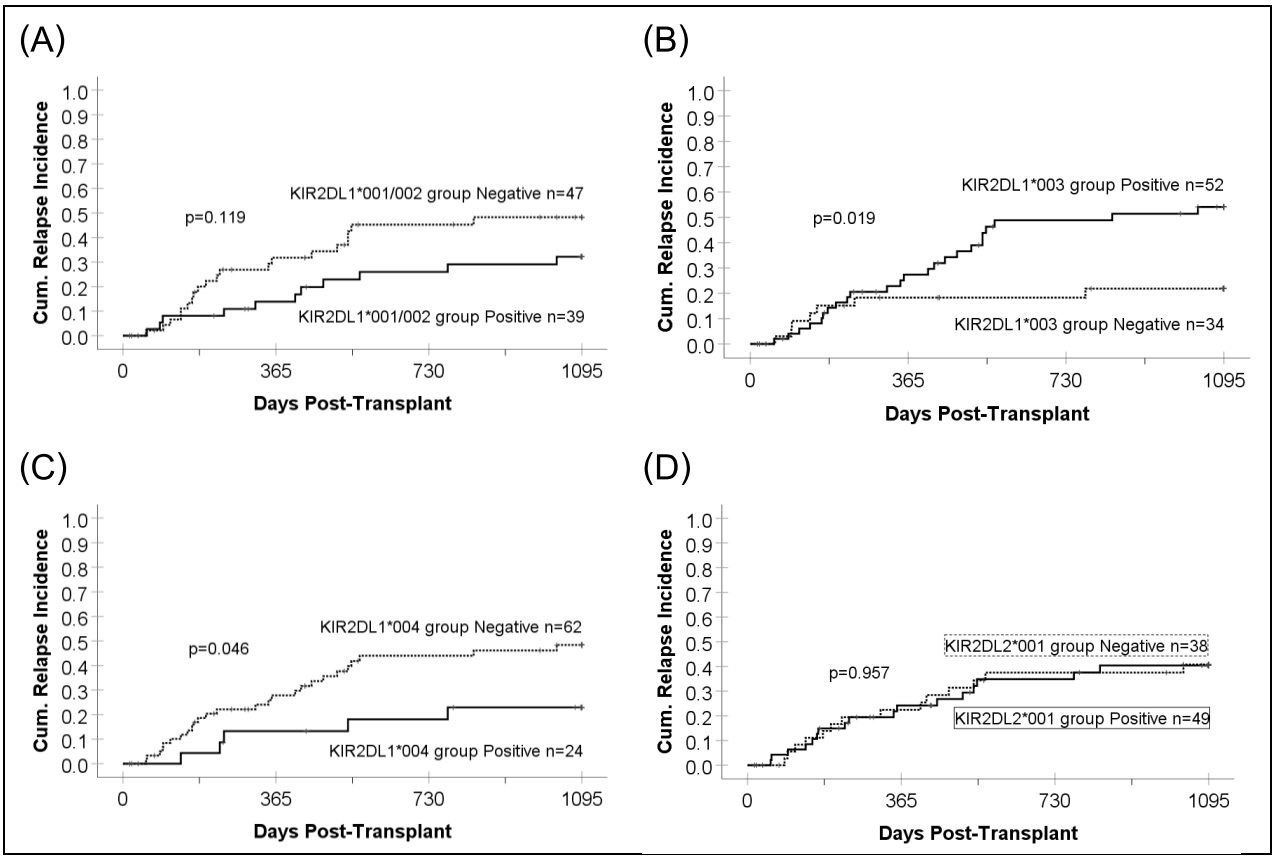


Figure 31 – The impact of donor KIR2DL1 (n=86) and KIR2DL2 (n=87) allele group presence/absence upon 3-year relapse incidence in T cell depleted RIC HPCT (Cohort B).

(A) Transplants using KIR2DL1*001/002 group presence/absence in the donor did not significantly influence incidence of relapse (p=0.119) (B) Transplants using KIR2DL1*003 group positive donors increased 3-year relapse incidence (p=0.019, p_c=0.0.90). (C) Transplants using KIR2DL1*004 group positive donors were significantly protective against 3-year post-transplant relapse incidence (p=0.046, p_c=0.184) (D) KIR2DL2*001 group presence/absence in the donor did not influence relapse incidence (p=0.957)

An advantageous effect was observed in KIR2DL1*004 group, with KIR2DL1*004 group positive donor transplants resulting in 22.9% compared to 48.4% in negative donor transplants ($p=0.046$, $p_c=0.184$).

KIR2DL1*001/002 and KIR2DL2*001 groups did not influence risk of relapse in the first 3 years post-transplant. KIR2DL1*001/002 group positive donor transplants displayed a divergence in 3-year relapse rates of 32.1% in comparison to 48.3% in transplants using donors negative for this allele group, but this did not reach significance ($p=0.119$). KIR2DL2*001 group presence/absence in the donor displayed little divergence, with both KIR2DL2*001 group positive and negative donor transplants presenting relapse rates of approximately 40.5% (40.4% and 40.8% respectively, $p=0.957$).

In the all transplants cohort, no donor KIR allele groups investigated significantly influenced risk of relapse post-transplant. The most significant finding in this cohort was the donor presence/absence of KIR2DL1*004 group. This allele group trended towards improving relapse rates, with 42.6% 3-year relapse in KIR2DL1*004 group positive donors versus 22.4% in transplants using donors negative for this allele group. No other allele groups investigated approached significance.

5.3.7 Graft versus Host Disease

KIR2DL2*001 donors trended towards increasing the incidence of GvHD grade II-IV. KIR2DL2*001 positive donor transplants displayed 3-year GvHD grade II-IV rate of 53.5%, compared to 30.2% in negative donor transplants ($p=0.062$, $p_c=0.372$). No other investigated KIR allele group displayed influence upon GvHD grade II-IV incidence. Divergence was observed in the KIR2DL1*004 group, with KIR2DL1*004 group positive donor transplants presenting a rate of 58.9% 3-year

GvHD grade II-IV, compared to 36.5% in negative donor transplants, but this did not approach significance (p=0.129).

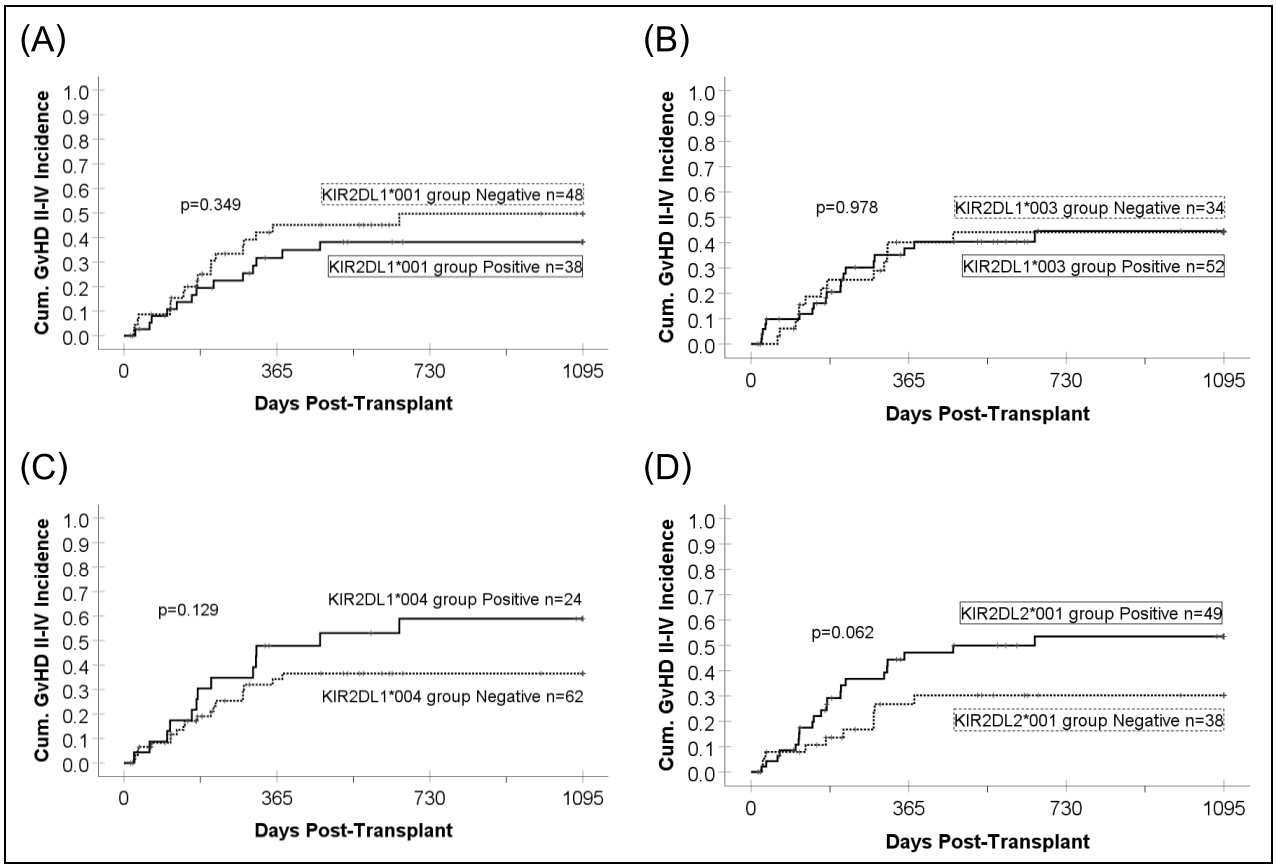


Figure 32 – The impact of donor KIR2DL1 (n=86) and KIR2DL2/3 (n=87) allele group presence/absence upon 3-year post-transplant graft versus host disease (GvHD) grade II-IV in T cell depleted RIC HPCT (Cohort B).

(A) Transplants using KIR2DL1*001/002 group positive/negative donors (p=0.349) (B) Transplants using KIR2DL1*003 group positive/negative donors (p=0.978). (C) Transplants using KIR2DL1*004 group positive/negative donors. No KIR2DL1 allele group in the donor was associated with incidence of 3-year post-transplant GvHD grade II-IV (p=0.129). (D) Transplants using KIR2DL2*001 group positive donors presented a non-significant trend towards increasing incidence of GvHD grade II-IV (p=0.062, p_c=0.372).

Similar findings were observed in Cohort A. The detrimental influence of KIR2DL2*001 group in donors reached significance, presenting 55.1% in KIR2DL2*001 group positive donor transplants versus 32.6% in negative donors (p=0.046, p_c=0.276) (HR=1.90, 95% CI=1.00-3.61, p=0.050, p_c=0.200).

5.3.8 Excluding influence of KIR A/A and B/x diplotypes

With allele groups displaying close association with KIR diplotype profiles, it is important to exclude the impact KIR diplotypes (and by extension KIR gene

presence/absence) exert upon post-transplant outcomes, particularly the difference between KIR A/A and B/x diplotypes possessed by the donor. To investigate the influence of KIR allele groups independently of KIR A/A versus B/x diplotype profiles, all described significant or approaching significance ($p<0.010$) findings in Cohort B were repeated, investigating only the transplants within this cohort that used KIR B/x diplotype donors (KIR2DL1 n=63, KIR2DL2 n=64).

All findings in survival (both OS and EFS) and relapse preserved significance in the KIR B/x donor sub-cohort. KIR2DL1*001/002 group positive donor transplants presented improved 3-year OS and EFS, with OS of 65.4% versus 29.7% in negative donor transplants ($p=0.013$), and EFS of 57.7% (positive donor) versus 24.3% (negative donor) ($p=0.013$). KIR2DL1*004 group positive donors presented comparable benefits in OS and EFS, with 62.2% 3-year OS (positive donor transplants) versus 33.3% (negative donor transplants) ($p=0.025$), and 3-year EFS of 54.2% (positive donor transplants) versus 28.2% (negative donor transplants) ($p=0.025$).

The detrimental effect of donor KIR2DL1*003 group upon OS and EFS were also maintained. The 3-year OS for transplants using KIR2DL1*003 group positive donors was 31.4% versus 60.7% in KIR2DL1*003 group negative donor transplants ($p=0.043$). KIR2DL1*003 positive donor transplants demonstrate 3-year EFS of 22.9% versus 57.1% in negative donor transplants ($p=0.012$).

The trend towards a detrimental impact of KIR2DL2*001 group positive donors in GvHD grade II-IV incidence was lost in the KIR B/x diplotype donor sub-cohort. KIR2DL2*001 positive donor transplants demonstrated 53.5% 3-year GvHD grade II-IV, compared to 47.4% in negative donor transplants ($p=0.706$).

It is not possible to further filter on Cen B/B donors, as demonstrated by the earlier findings that KIR2DL1*001/002 and 003 are associated with KIR CenA ($KIR2DL3^{+}$) haplotypes and KIR2DL1*004 is associated with KIR CenB ($KIR2DL2^{+}$) haplotypes.

5.4 Discussion

Previous studies have demonstrated the important influence of KIR allelic polymorphism upon transplant outcomes (Bari et al., 2013; Boudreau et al., 2017). Anecdotally, the KIR genomics community believe conditioning regimes and work-up regime are integral to taking advantage of NK cell genomics, due to conditioning helping to establish the environment for immune reconstitution and NK cells representing the first lymphocytes to reconstitute post-transplant (Ullah et al., 2016; Falco et al., 2019). The study contributes to the body of work exploring the impact of KIR genomics upon HPCT outcomes, and verifies the suggestions that the conditioning regime changes the transplant outcomes observed in the context of NK cell genomics.

Strong linkage was demonstrated between KIR2DL1 and KIR2DL2/3 allele groups, negating the value in independent analysis of all genes encoding KIR glycoproteins specific for C1 and C2 epitopes. Strong linkage was observed between KIR2DL1*001/002 and KIR2DL3*002 groups, and between KIR2DL1*003 and KIR2DL3*001 groups (Figure 27). Moderate linkage was observed between KIR2DL1*004 and KIR2DL2*001 groups, with a strong NPV (58.1%), but a weaker PPV (95.8%). Despite the high level of recombinant events occurring within the KIR gene cluster, this close association may be expected due to all three genes being located within the centromeric region of the KIR gene cluster (Martin et al., 2003; Norman et al., 2009). The observed restriction of KIR2DL1/2/3 allele group

combinations within each KIR diplotype identity suggests that the KIR allele groups are likely inherited as a relatively conserved set (Gourraud et al., 2010; Pyo et al., 2013). Despite this observed restriction, the loose association of KIR2DL1/2/3 allele groups with KIR diplotype identities indicates that KIR diplotype identities cannot be used as the sole proxy or predictor of the respective KIR2DL1/2/3 allele groups possessed.

The KIR2DL1 residues explored in this study have been well-characterised. Positions 114 and 245 are located within exon 5 and 7 respectively. Exon 5 encodes the D2 region, influencing the overall avidity of the receptor. KIR2DL1-P^{114} receptors (KIR2DL1*001/002 and 004 groups) provide stronger binding avidity than KIR2DL1-L^{114} (KIR2DL1*003 group) (Hilton et al., 2015a). Exon 7 encodes the transmembrane region, influencing signalling capacity of the receptor. KIR2DL1-R^{245} receptors (KIR2DL1*001/002 and 003 groups) possess stronger signalling capacity than KIR2DL1-C^{245} (KIR2DL1*004 group) (Bari et al., 2011; Bari et al., 2013; Hilton et al., 2015a).

Despite the well characterised features of allelic polymorphism in KIR2DL1, the basic scientific principles underlying the observed detrimental effects of donors possessing the KIR2DL1*003 group compared to KIR2DL1*001/002 and 004 groups remains unclear. KIR2DL1*001 and 003 are highly homologous in DNA sequence, differing by a single non-synonymous mutation at codon 114, and three synonymous mutations at codons 16, 27, and 216. In contrast, the KIR2DL1*004 group differs greatly from other KIR2DL1 alleles, exhibiting partial homology with the activating KIR gene, KIR2DS1 (Maccari et al., 2020). The avidity of KIR2DL1 is largely determined by four residues within the D2 domain, encoded within exon 5: positions 114, 154, 163,

and 182, with position 114 presenting the greatest influence (Hilton et al., 2015a). Despite P^{114} generally displaying increased receptor avidity compared to L^{114}, KIR2DL1*003 group alleles (L^{114}, P^{154}, D^{163}, H^{182}) are characterised by its overall high avidity compared to that of KIR2DL1*004 group alleles (P^{114}, T^{154}, N^{163}, R^{182}) (Hilton et al., 2015a). Though the overall avidity of KIR2DL1*001 was not ascertained, substituting P^{114} into KIR2DL1*003 to match the secondary structure of KIR2DL1*001 increased the avidity further. This suggests there is not a specific KIR2DL1 avidity profile that correlates with post-transplant outcome, with both the high avidity of KIR2DL1*001 group and low avidity of KIR2DL1*004 group both improving post-transplant outcomes.

The observed advantage of donor possessing the KIR2DL1*001/002 and 004 groups in transplantation outcomes were of a comparable magnitude, but differ in their position 245 amino acid residue, suggesting the signalling capacity of the KIR2DL1 receptor may have a negligible influence in HPCT outcomes. Further work is required to fully assess the influence of these two dimorphic positions upon transplant outcomes, particularly into the effects of donors possessing homozygosity or heterozygosity at these positions.

Previous findings of KIR2DL1 polymorphisms identified conflicting transplant outcomes when compared to the results of the study. In opposition to this study's findings, Bari et al. (2013) identified KIR2DL1-R^{245} in association with improved OS, and KIR2DL1-C^{245} as a neutral factor, with no difference in survival between KIR2DL1-R/R^{245} and KIR2DL1-R/C^{245} donors. These results were in the context of a majority myeloablative conditioning (76.7% MAC versus 23.3% RIC), and a mix of T cell depleted (49.2%) and T cell replete (50.8%) transplant workup regimes. The

opposing findings to this study may be caused by the variance of transplant treatment protocols. With NK cells being the first lymphocyte to reconstitute post-transplant, detectable within 14 days post-transplant, it would be logical to presume that NK cells are likely to be sensitive to the cytotoxicity of conditioning regime and the presence or absence of donor T cells in the immediate period post-transplant (Baron and Sandmaier, 2006; Chan et al., 2018). This suggestion was enforced by a recent study which found opposing findings in myeloablative and RIC conditioning, and has been discussed amongst the KIR research community at the recent International KIR Workshop (Bultitude et al., 2018; Falco et al., 2019).

Hilton et al. (2015a) postulated that the centromeric regions of KIR A haplotypes (CenA) are characterised by low avidity C1-specific inhibitory KIR (encoded by KIR2DL3) in association with high avidity C2-specific inhibitory KIR (encoded by KIR2DL1*001/002 and 003 groups), and centromeric regions of KIR B haplotypes (CenB) are characterised by and high avidity C1-specific inhibitory KIR (encoded by KIR2DL2) in association with low avidity C2-specific inhibitory KIR (encoded by KIR2DL1*004 group). These linkage relationships were verified in the investigated cohort data (Figure 26). The balance of C1 and C2 receptor avidity in turn exerts evolutionary pressures on the basis of a population's C1 and C2 epitope frequencies, which is highly variable between populations (Hilton et al., 2015a). This suggests KIR allele groups may act as an indicator of wider variance due to these evolutionary pressures, with deeper implications for the NK cell immune response.

It is of note that the analysis has not considered the HLA type of the recipient or donor, and has therefore identified the significance of KIR allelic polymorphism in post-HPCT outcomes irrespective of the KIR licensing environment. Unlicensed NK cells

are considered hyporesponsive to stimulus, and so licensing may be expected to have a significant impact upon the NK cell immune response post-transplant (Anfossi et al., 2006). Licensing may have nominal influence upon the impact of KIR2DL1/2/3 allelic polymorphism, or HPCT outcomes could be further improved by consideration of the NK cell licensing process. The influence of the KIR receptor and ligand relationship in licensing in the context of KIR2DL1/2/3 allelic polymorphism would be a worthwhile subject for further study.

This study, along with the related findings of others, suggests the approach of using assessment of KIR genomics within HPCT protocols may require reassessment at a fundamental level. The lack of universal consistency across all transplant centres in the effectiveness of published clinical KIR assessment algorithms in HPCT may be in part due to the heterogeneity in conditioning protocols and other variations in therapeutic protocols (Schetelig et al., 2020; Verneris et al., 2020). Finer details of the transplantation protocol, such as the form of T cell depletion (e.g. Alemtuzumab, ATG, or high dose Cyclophosphamide), may also impact upon the effectiveness of KIR assessment algorithms. Once the effect of broader variation in protocols is understood, the scientific community will be better placed to then investigate more subtle variation.

Research into the relationship between KIR genetics and proteomics is essential for translational clinical research, providing a scientific rationale for stratifying individuals in clinical trials on the basis of KIR genetic data. The study has demonstrated the benefits of donors possessing KIR2DL1*001/002 and/or KIR2DL1*004 groups, and the detrimental effect of donors possessing the

KIR2DL1*003 group, in T cell depleted RIC HPCT. KIR2DL1*003 negative donors offer improvements to 3-year relapse incidence, OS, and EFS.

Chapter 5 References

Alicata, C., Pende, D., Meazza, R., Canevali, P., Loiacono, F., Bertaina, A., Locatelli, F., Nemat-Gorgani, N., Guethlein, L. A., Parham, P., Moretta, L., Moretta, A., Bottino, C., Norman, P. J. & Falco, M. (2016) Hematopoietic stem cell transplantation: Improving alloreactive Bw4 donor selection by genotyping codon 86 of KIR3DL1/S1. *Eur J Immunol,* 46(6)**,** 1511-7.

Anfossi, N., Andre, P., Guia, S., Falk, C. S., Roetynck, S., Stewart, C. A., Breso, V., Frassati, C., Reviron, D., Middleton, D., Romagne, F., Ugolini, S. & Vivier, E. (2006) Human NK cell education by inhibitory receptors for MHC class I. *Immunity,* 25(2)**,** 331-42.

Arima, N., Kanda, J., Tanaka, J., Yabe, T., Morishima, Y., Kim, S. W., Najima, Y., Ozawa, Y., Eto, T., Kanamori, H., Mori, T., Kobayashi, N., Kondo, T., Nakamae, H., Uchida, N., Inoue, M., Fukuda, T., Ichinohe, T., Atsuta, Y. & Kanda, Y. (2018) Homozygous HLA-C1 is associated with reduced risk of relapse after HLA-matched transplantation in patients with myeloid leukemia. *Biol Blood Marrow Transplant,* 24(4)**,** 717-25.

Bari, R., Bell, T., Leung, W. H., Vong, Q. P., Chan, W. K., Das Gupta, N., Holladay, M., Rooney, B. & Leung, W. (2009) Significant functional heterogeneity among KIR2DL1 alleles and a pivotal role of arginine 245. *Blood,* 114(25)**,** 5182-90.

Bari, R., Leung, M., Turner, V. E., Embrey, C., Rooney, B., Holladay, M. & Leung, W. (2011) Molecular determinant-based typing of KIR alleles and KIR ligands. *Clin Immunol,* 138(3)**,** 274-81.

Bari, R., Rujkijyanont, P., Sullivan, E., Kang, G., Turner, V., Gan, K. & Leung, W. (2013) Effect of donor KIR2DL1 allelic polymorphism on the outcome of pediatric allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol,* 31(30)**,** 3782-90.

Baron, F. & Sandmaier, B. M. (2006) Chimerism and outcomes after allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning. *Leukemia,* 20(10)**,** 1690-700.

Boudreau, J. E., Giglio, F., Gooley, T. A., Stevenson, P. A., Le Luduec, J. B., Shaffer, B. C., Rajalingam, R., Hou, L., Hurley, C. K., Noreen, H., Reed, E. F., Yu, N., Vierra-Green, C., Haagenson, M., Malkki, M., Petersdorf, E. W., Spellman, S. & Hsu, K. C. (2017) KIR3DL1/HLA-B Subtypes Govern Acute Myelogenous Leukemia Relapse After Hematopoietic Cell Transplantation. *J Clin Oncol,* 35(20)**,** 2268-2278.

Boudreau, J. E., Le Luduec, J. B. & Hsu, K. C. (2014) Development of a novel multiplex PCR assay to detect functional subtypes of KIR3DL1 alleles. *PLoS One,* 9(6)**,** e99543.

Bultitude, W. P., Gymer, A. W., Robinson, J., Anthias, C., Potter, M. N., Russell, N., Pagliuca, A., Potter, V. T., Bloor, A. J., Marks, D., Patel, A., MacKinnon, S., Carpenter, B., McQuaker, I. G., Szydlo, R. M., Perry, J., Wilson, M., Mayor, N. P. & Marsh, S. G. E. (Year) The effect of donor KIR2DL1 allelic diversity on the outcomes of HSCT is influenced by conditioning regimen. *In:* KIR Workshop 25th-27th October 2018 21/07/2019 2018 Camogli (GE) Italy. HLA.

Chan, Y. L. T., Zuo, J., Inman, C., Croft, W., Begum, J., Croudace, J., Kinsella, F., Maggs, L., Nagra, S., Nunnick, J., Abbotts, B., Craddock, C., Malladi, R. &

Moss, P. (2018) NK cells produce high levels of IL-10 early after allogeneic stem cell transplantation and suppress development of acute GVHD. *Eur J Immunol,* 48(2), 316-329.

Colonna, M., Spies, T., Strominger, J. L., Ciccone, E., Moretta, A., Moretta, L., Pende, D. & Viale, O. (1992) Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene. *Proc Natl Acad Sci U S A,* 89(17), 7983-5.

Falco, M., Sivori, S., Meazza, R. & Pende, D. (2019) Report from the Eleventh Killer Immunoglobulin-like Receptor (KIR) Workshop: Novel insights on KIR polymorphism, ligand recognition, expression and function. *HLA,* 94(2), 100-110.

Forlenza, C. J., Boudreau, J. E., Zheng, J., Le Luduec, J. B., Chamberlain, E., Heller, G., Cheung, N. K. & Hsu, K. C. (2016) KIR3DL1 Allelic Polymorphism and HLA-B Epitopes Modulate Response to Anti-GD2 Monoclonal Antibody in Patients With Neuroblastoma. *J Clin Oncol,* 34(21), 2443-51.

Gaetano, J. (2018) *Holm-Bonferroni sequential correction: An Excel calculator (1.3) [Microsoft Excel Workbook]* [Online]. Available: https://www.researchgate.net/publication/322568540_Holm-Bonferroni_sequential_correction_An_Excel_calculator_13 [Accessed].

Gourraud, P. A., Meenagh, A., Cambon-Thomsen, A. & Middleton, D. (2010) Linkage disequilibrium organization of the human KIR superlocus: implications for KIR data analyses. *Immunogenetics,* 62(11-12), 729-40.

Hansasuta, P., Dong, T., Thananchai, H., Weekes, M., Willberg, C., Aldemir, H., Rowland-Jones, S. & Braud, V. M. (2004) Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. *Eur J Immunol,* 34(6), 1673-9.

Hilton, H. G., Guethlein, L. A., Goyos, A., Nemat-Gorgani, N., Bushnell, D. A., Norman, P. J. & Parham, P. (2015a) Polymorphic HLA-C receptors balance the functional characteristics of KIR haplotypes. *J Immunol,* 195(7), 3160-70.

Le Luduec, J. B., Boudreau, J. E., Freiberg, J. C. & Hsu, K. C. (2019) Novel Approach to Cell Surface Discrimination Between KIR2DL1 Subtypes and KIR2DS1 Identifies Hierarchies in NK Repertoire, Education, and Tolerance. *Front Immunol,* 10, 734.

Maccari, G., Robinson, J., Hammond, J. A. & Marsh, S. G. E. (2020) The IPD Project: a centralised resource for the study of polymorphism in genes of the immune system. *Immunogenetics,* 72(1-2), 49-55.

Mandelboim, O., Reyburn, H. T., Valés-Gómez, M., Pazmany, L., Colonna, M., Borsellino, G. & Strominger, J. L. (1996) Protection from lysis by Natural Killer cells of group 1 and 2 specificity is mediated by residue 80 in human Histocompatibility Leukocyte Antigen C alleles and also occurs with empty Major Histocompatibility Complex molecules. *J Exp Med,* 184, 913-22.

Martin, M. P., Bashirova, A., Traherne, J., Trowsdale, J. & Carrington, M. (2003) Cutting edge: expansion of the KIR locus by unequal crossing over. *The Journal of Immunology,* 171(5), 2192-5.

Moesta, A. K., Norman, P. J., Yawata, M., Yawata, N., Gleimer, M. & Parham, P. (2008) Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *The Journal of Immunology,* 180(6), 3969-79.

Mulrooney, T. J., Hou, L., Steiner, N. K., Chen, M., Belle, I., Ng, J. & Hurley, C. K. (2008) Promoter variants of KIR2DL5 add to diversity and may impact gene expression. *Immunogenetics,* 60(6), 287-94.

Neuchel, C., Furst, D., Niederwieser, D., Bunjes, D., Tsamadou, C., Wulf, G., Pfreundschuh, M., Wagner, E., Stuhler, G., Einsele, H., Schrezenmeier, H. & Mytilineos, J. (2017) Impact of donor activating KIR genes on HSCT outcome in C1-ligand negative myeloid disease patients transplanted with unrelated donors - a retrospective study. *PLoS One,* 12(1), e0169512.

Norman, P. J., Abi-Rached, L., Gendzekhadze, K., Hammond, J. A., Moesta, A. K., Sharma, D., Graef, T., McQueen, K. L., Guethlein, L. A., Carrington, C. V., Chandanayingyong, D., Chang, Y. H., Crespi, C., Saruhan-Direskeneli, G., Hameed, K., Kamkamidze, G., Koram, K. A., Layrisse, Z., Matamoros, N., Mila, J., Park, M. H., Pitchappan, R. M., Ramdath, D. D., Shiau, M. Y., Stephens, H. A., Struik, S., Tyan, D., Verity, D. H., Vaughan, R. W., Davis, R. W., Fraser, P. A., Riley, E. M., Ronaghi, M. & Parham, P. (2009) Meiotic recombination generates rich diversity in NK cell receptor genes, alleles, and haplotypes. *Genome Res,* 19(5), 757-69.

O'Connor, G. M., Vivian, J. P., Gostick, E., Pymm, P., Lafont, B. A., Price, D. A., Rossjohn, J., Brooks, A. G. & McVicar, D. W. (2015) Peptide-dependent recognition of HLA-B*57:01 by KIR3DS1. *J Virol,* 89(10), 5213-21.

Pyo, C. W., Wang, R., Vu, Q., Cereb, N., Yang, S. Y., Duh, F. M., Wolinsky, S., Martin, M. P., Carrington, M. & Geraghty, D. E. (2013) Recombinant structures expand and contract inter and intragenic diversification at the KIR locus. *BMC Genomics,* 14, 89.

Schetelig, J., Baldauf, H., Heidenreich, F., Massalski, C., Frank, S., Sauter, J., Stelljes, M., Ayuk, F. A., Bethge, W. A., Bug, G., Klein, S., Wendler, S., Lange, V., de Wreede, L. C., Furst, D., Kobbe, G., Ottinger, H. D., Beelen, D. W., Mytilineos, J., Fleischhauer, K., Schmidt, A. H. & Bornhauser, M. (2020) External validation of models for KIR2DS1/KIR3DL1-informed selection of hematopoietic cell donors fails. *Blood,* 135(16), 1386-1395.

Sharma, D., Bastard, K., Guethlein, L. A., Norman, P. J., Yawata, N., Yawata, M., Pando, M., Thananchai, H., Dong, T., Rowland-Jones, S., Brodsky, F. M. & Parham, P. (2009) Dimorphic motifs in D0 and D1+D2 domains of killer cell Ig-like receptor 3DL1 combine to form receptors with high, moderate, and no avidity for the complex of a peptide derived from HIV and HLA-A*2402. *J Immunol,* 183(7), 4569-82.

Shimoni, A., Vago, L., Bernardi, M., Yerushalmi, R., Peccatori, J., Greco, R., Shem-Tov, N., Lo Russo, A., Danylesko, I., Apel, A., Bonini, C., Lupo Stanghellini, M. T., Nagler, A. & Ciceri, F. (2017) Missing HLA C group 1 ligand in patients with AML and MDS is associated with reduced risk of relapse and better survival after allogeneic stem cell transplantation with fludarabine and treosulfan reduced toxicity conditioning. *Am J Hematol,* 92(10), 1011-1019.

Sim, M. J., Malaker, S. A., Khan, A., Stowell, J. M., Shabanowitz, J., Peterson, M. E., Rajagopalan, S., Hunt, D. F., Altmann, D. M., Long, E. O. & Boyton, R. J. (2017) Canonical and Cross-reactive Binding of NK Cell Inhibitory Receptors to HLA-C Allotypes Is Dictated by Peptides Bound to HLA-C. *Front Immunol,* 8, 193.

Uhrberg, M. (2005) Shaping the human NK cell repertoire: an epigenetic glance at KIR gene regulation. *Mol Immunol,* 42(4), 471-5.

Ullah, M. A., Hill, G. R. & Tey, S. K. (2016) Functional Reconstitution of Natural Killer Cells in Allogeneic Hematopoietic Stem Cell Transplantation. *Front Immunol,* 7, 144.

VandenBussche, C. J., Dakshanamurthy, S., Posch, P. E. & Hurley, C. K. (2006) A single polymorphism disrupts the killer Ig-like receptor 2DL2/2DL3 D1 domain. *J Immunol,* 177(8), 5347-57.

Verneris, M. R., Miller, J. S., Hsu, K. C., Wang, T., Sees, J. A., Paczesny, S., Rangarajan, H., Lee, D. A., Spellman, S. R. & Lee, S. J. (2020) Investigation of donor KIR content and matching in children undergoing hematopoietic cell transplantation for acute leukemia. *Blood Adv,* 4(7), 1350-1356.

Winter, C. C., Gumperz, J. E., Parham, P., Long, E. O. & Wagtmann, N. (1998) Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *The Journal of Immunology,* 161, 571-77.

Chapter 6 | Constructing a novel algorithm based upon donor KIR2DL1 single nucleotide polymorphism motifs in donor selection for T cell depleted reduced intensity conditioning haematopoietic progenitor cell transplantation

Summary

The detrimental impact of donors positive for KIR2DL1*003 allele groups in the setting of T cell depleted reduced intensity conditioning (RIC) HPCT was previously described, with a corresponding improvement in post-transplant outcomes with donors possessing KIR2DL1*001/002 and/or 004 allele group donors. Although next generation sequencing is an effective means of defining KIR alleles, it is an expensive technique that places financial and workload pressures upon clinical laboratories, reducing the likelihood of routine implementation of KIR donor assessment. In an effort to potentially reduce the cost of the requisite testing, the influence of the single nucleotide polymorphisms (SNPs) used to define the allele groups, positions 114 and 245, were further explored. Donors possessing proline at position 114 of KIR2DL1 (KIR2DL1-P^{114}) improved 3-year overall survival (OS) (58.8% versus 28.6%, p=0.008), event-free survival (EFS) (51.0% versus 25.7%, p=0.018), and reduced incidence of relapse (31.5% versus 54.9%, p=0.028) compared to donors negative for this characteristic. In contrast, transplants using donors with leucine at this position (KIR2DL1-L^{114}) experienced decreased 3-year OS (36.5% versus 61.8%, p=0.046), EFS (28.9% versus 58.8%, p=0.015), and increased relapse incidence (54.1% versus 21.9%, p=0.019). Donors possessing cysteine at position 245 (KIR2DL1-C^{245}) correlated with a trend towards improved post-transplant outcome in OS (66.7% versus 40.3%, p=0.066), EFS (54.2% versus 35.5%, p=0.077), and significant reduction in relapse incidence (22.9% versus 48.4%, p=0.046). Arginine at this position (KIR2DL1-R^{245}) in the donor was found to be neutral, with no effect on the investigated transplant outcomes. Despite the relatively small sample size, donors homozygous for KIR2DL1-P^{114} appeared to present as the optimal donor, with significantly improved 3-year OS (66.7% versus 29.0%, p=0.006) and reduced relapse incidence (17.8% versus 52.3%, p=0.031) compared to transplants using KIR2DL1-L/P^{114} heterozygous donors. These findings support the reasoning for developing an inexpensive technique for defining signature SNP motifs as a means of assessing KIR allele groups, such as a real-time PCR or sequence specific primer (SSP) kit. Further investigation with a larger cohort would be valuable to determine the influence of KIR2DL1-P/L^{114} and -C/R^{245} homozygosity and heterozygosity in higher detail, and to determine whether parallel assessment of KIR2DL1 positions 114 and 245 in potential donors provides additional value compared to assessing KIR2DL1 position 114 alone.

6.1 Introduction

The influence of KIR allele groups upon haematopoietic progenitor cell transplantation (HPCT) has been previously demonstrated (Chapter 5). Three KIR2DL1 allele groups were described: KIR2DL1*001/002, 003, and 004 groups. Transplants using donor grafts possessing KIR2DL1*001/002 and/or 004 groups exhibit significantly reduced relapse incidence, and improved overall (OS) and event-free survival (EFS) compared to donors possessing the KIR2DL1*003 group (Chapter 5). The benefits were shown to be substantial, with 21.9% versus 54.1% 3-year relapse incidence in transplants using KIR2DL1*003 group negative donors and positive donors respectively (p=0.019). Similar improvements in OS are observed, with 61.8% versus 36.5% 3-year OS in KIR2DL1*003 group negative donors and positive donors respectively (p=0.017). KIR2DL1*003 group positive donor transplants represent hazard ratio (HR) of 2.65 for relapse incidence (95% CI=1.14-6.17, p=0.024) and 1.91 for all-cause mortality (95% CI=1.00-3.63, p=0.050) compared to KIR2DL1*003 negative donor transplants.

One of the largest obstacles to overcome when proposing a novel test to the clinical community are the financial and workload burden in taking on any novel procedure. HPCT is one of the most expensive medical practices carried out, with reduced intensity conditioning (RIC) transplants costing a mean price of $300,871 per transplant in the United States (2017 valuation) (Khera et al., 2012; Broder et al., 2017). Several factors already considered in selecting the optimal donor, including donor HLA allele compatibility with the recipient, donor age, donor cytomegalovirus (CMV) serostatus matching with the recipient, and additional assessments of donor HLA genetics (e.g. HLA-DPB1 reactivity) (Petersdorf et al., 2004; Fleischhauer et al.,

2014; Shaw et al., 2017; Shaw et al., 2018; Mayor et al., 2019). Consequently, the cost of any further testing, such as KIR genotyping, must be as low as possible to encourage the wider histocompatibility and immunogenetics (H&I) community to consider implementing the test into routine service.

This study acts as an addendum to the findings reported in Chapter 5, interrogating how each of the constituent amino acid markers of KIR2DL1 allele groups (KIR2DL1-P/L^{114} and R/C^{245}) influenced transplant outcomes. The ultimate aim was to assess how a clinical model based upon identification of single nucleotide polymorphism (SNPs) could be viable as a substitute for defining KIR2DL1 allele groups. This data would verify if an inexpensive technology, such as PCR-Sequence-specific primers (PCR-SSP) or real-time PCR (qPCR), could be used to predict optimal post-transplant outcomes in place of costly KIR gene sequencing by next generation sequencing (NGS).

6.2 Methods

6.2.1 Cohort Selection

The cohort selection protocol is described in Chapter 4.2.1.

The inclusion and exclusion criteria was identical to that described in Chapter 4.2.1. Two further transplant pairs were excluded from the study due to failure of KIR2DL1 gene sequencing for the respective donor.

The final cohort sizes were as follows: Cohort A (n=113), and Cohort B (n=86).

6.2.2 KIR Genotyping & Definition

Amplicon-based short-read NGS of KIR was carried out, as described in Chapter 5.2.2.

Translation of codons 114 and 245 from DNA sequence were translated to their corresponding amino acid residues for means of KIR2DL1 grouping definition. Both positions were dimorphic in nature, with the KIR2DL1 grouping described as KIR2DL1-X^{YYY}, where X represents the encoded residue and YYY represents the residue position (e.g. KIR2DL1-P^{114} = KIR2DL1 glycoproteins with proline at position 114).

6.2.3 Statistical Analysis

Frequencies for each characteristic were calculated according to proportional representation of each respective type with the sample size. All Kaplan Meier survival curves and hazard ratios (HR) were calculated using IBM SPSS Statistics v25. Kaplan Meier survival curves were constructed for primary and secondary transplant outcomes factors, with log rank regression and cox regression (with associated hazard ratio data) also calculated. Statistical significance was considered to be $p \leq 0.05$. Holm-Bonferonni sequential correction was carried out for all statistics to provide a corrected p value (p_c) (Gaetano, 2018). The p_c values are only presented where the respective $p<0.10$.

6.2.4 Transplant Outcome Definitions

Transplant outcome definitions are described in Chapter 4.2.4.

5.2.5 Ethics

Ethical approval is described in Chapter 4.2.5.

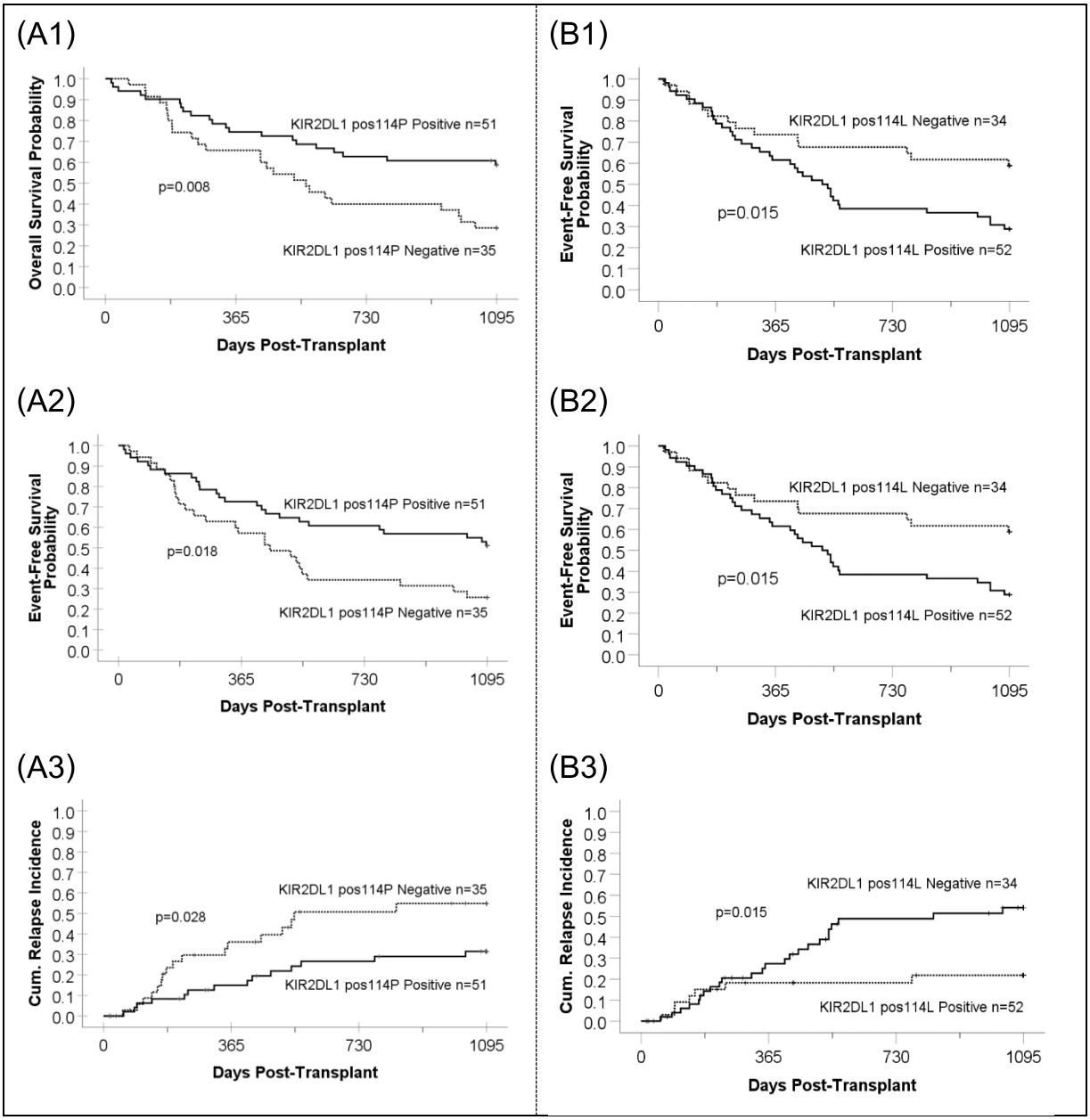


Figure 33 – The influence of KIR2DL1-P^{114} (left) and -L^{114} (right) presence/absence in the donor upon post-transplant survival outcomes in T cell depleted RIC HPCT (Cohort B) (n=86).

Presence of KIR2DL1-P^{114} in the donor improved 3-year post-transplant (A1) overall survival (OS) (p=0.008, p_c=0.032), (A2) event-free survival (EFS) (p=0.018, p_c=0.060), and (A3) reduced 3-year relapse incidence (p=0.028, p_c=0.084). Presence of KIR2DL1-L^{114} in the donor was detrimental to (B1) 3-year OS (p=0.046, p_c=0.138) and (B2) EFS (p=0.015, p_c=0.060), and (B3) increased incidence of relapse (p=0.019, p_c=0.076).

6.3 Results

6.3.1 Influence of KIR2DL1 position 114 and 245 upon transplant outcomes

Donor KIR2DL1 residues 114 and 245 were found to significantly influence post-HPCT outcomes (Table 28, Figure 33 and Figure 34).

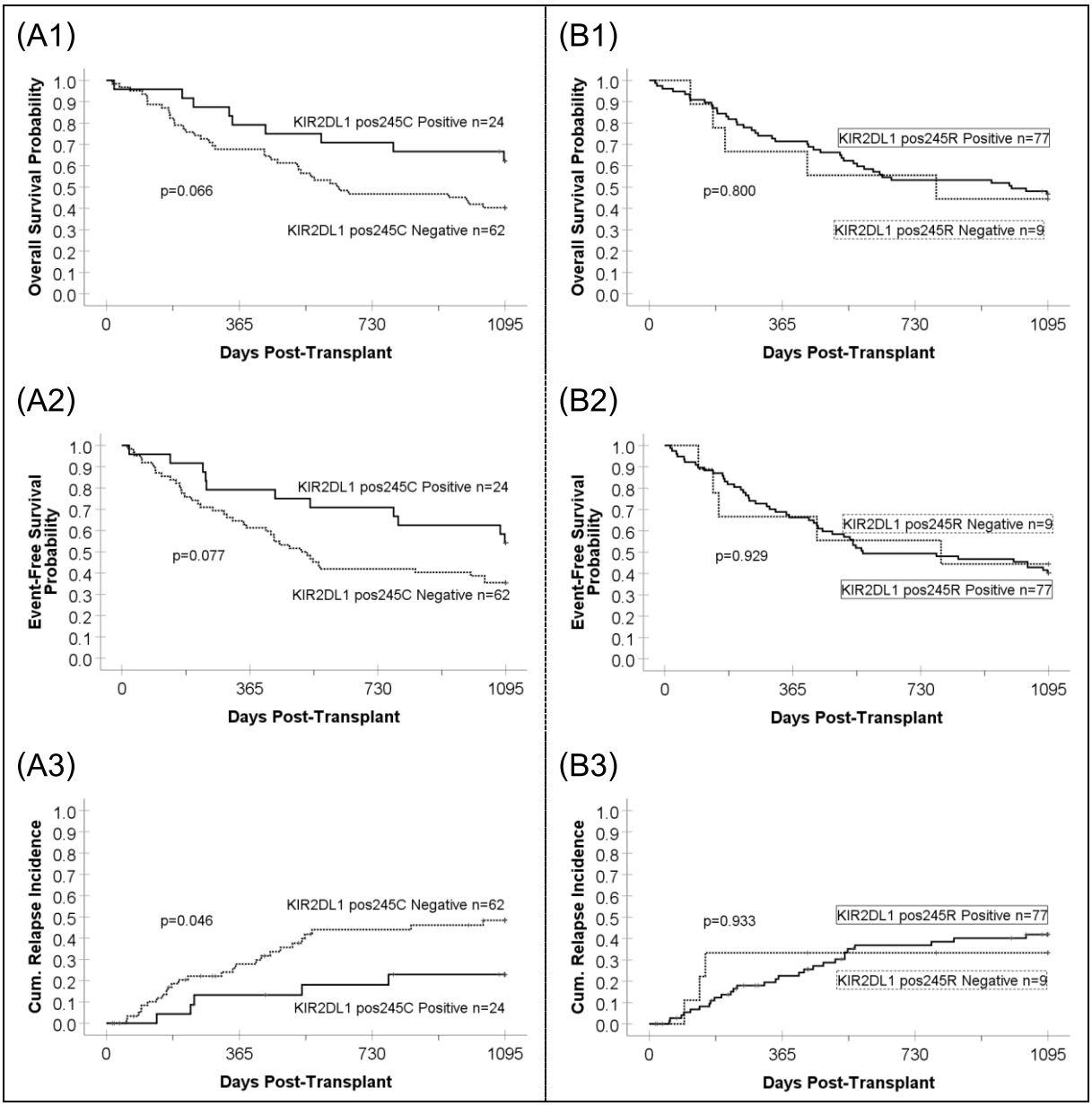


Figure 34 — The influence of KIR2DL1-C^{245} (left) and R^{245} (right) presence/absence in the donor upon post-transplant outcomes in T cell depleted RIC HPCT (Cohort B) (n=86).

Presence of KIR2DL1-C^{245} in the donor trended towards improving (A1) 3-year OS (p=0.066) and (A2) EFS (p=0.077), and (A3) significantly reduced 3-year post-transplant relapse incidence (p=0.046). Presence/absence of KIR2DL1-R^{245} in the donor did not influence (B1) OS (p=0.800), (B2) EFS (p=0.929), or (B3) relapse incidence (p=0.933)

Transplants using donors possessing KIR2DL1-P^{114} consistently exhibited significant beneficial effects in 3-year OS (58.8% versus 28.6%, p=0.008, p_c=0.032), EFS (51.0% versus 25.7%, p=0.018, p_c=0.060), and reduced incidence of relapse (31.5% versus 54.9%, p=0.028, p_c=0.084) (Table 28, Figure 33). KIR2DL1-L^{114} in the donor was

detrimental to post-transplant outcomes, with significantly detrimental effect upon 3-year OS (36.5% versus 61.8%, p=0.046, p_c=0.138), and EFS (28.9% versus 58.8%, p=0.015, p_c=0060), and an increased incidence of relapse (54.1% vs 21.9%, p=0.019, p_c=0.076).

decreased OS (36.5% vs 61.8%, p=0.046) and EFS (28.9% vs 58.8%, p=0.015) compared to KIR2DL1-L^{114} negative donor transplants. The HR of 3-year post-transplant all-cause mortality for KIR2DL1-P^{114} positive donor transplants compared to a KIR2DL1-P^{114} negative donor transplant is 0.46 (95% CI= 0.26-0.83, p=0.010, p_c=0.040). In contrast, the HR of 3-year post-transplant all-cause mortality for KIR2DL1-L^{114} positive donor transplant compared to a transplant using a KIR2DL1-L^{114} negative donor was 1.91 (95% CI=1.00-3.63, p=0.050, p_c=0.150) (Table 28).

KIR2DL1 Amino Acid Identity	Category	n (%)	All-Cause Mortality HR (95% CI)	Event HR (95% CI)	Relapse HR (95% CI)
P^{114}	Negative Positive	35 (40.7%) 51 (59.3%)	1 0.46 (0.26-0.83) (p=0.010, p_c=0.040)	1 0.52 (0.30-0.90) (p=0.020, p_c=0.068)	1 0.46 (0.23-0.94) (p=0.032, p_c=0.096)
L^{114}	Negative Positive	34 (39.5%) 52 (60.5%)	1 1.91 (1.00-3.63) (p=0.050, p_c=0.150)	1 2.12 (1.14-3.94) (p=0.017, p_c=0.068)	1 2.65 (1.14-6.17) (p=0.024, p_c=0.096)
C^{245}	Negative Positive	62 (72.1%) 24 (27.9%)	1 0.51 (0.25-1.06) (p=0.072, p_c=0.150)	1 0.55 (0.283-1.08) (p=0.082, p_c=0.164)	1 0.39 (0.15-1.02) (p=0.055, p_c=0.110)
R^{245}	Negative Positive	9 (10.5%) 77 (89.5%)	1 0.89 (0.35-2.25) (p=0.800)	1 1.04 (0.41-2.63) (p=0.930)	1 1.05 (0.32-3.46) (p=0.933)

Table 28 – Influence of KIR2DL1 polymorphism at positions 114 and 245 upon post-transplant outcomes in T cell depleted RIC HPCT (Cohort B) (n=86).

KIR2DL1-P^{114} and -C^{245} demonstrate beneficial influence upon 3-year post-transplant outcomes. In contrast, KIR2DL1-L^{114} exhibit detrimental effects upon HPCT outcomes. KIR2DL1-R^{245} appears neutral in all post-transplant outcomes assessed. **Key**: Data with p≤0.05 are shaded blue. Data with 0.05<p≤0.10 are shaded orange.

Transplants using donors possessing KIR2DL1-C^{245} trended towards improved outcomes in 3-year OS (66.7% versus 40.3%, p=0.066, p_c=0.066) and EFS (54.2% versus 35.5%, p=0.077, p_c=0.154), and presented significant reduction in rate of relapse (22.9% versus 48.4%, p=0.046, p_c=0.092) (Figure 34). Donor KIR2DL1-R^{245} was neutral in regards to post-HPCT outcome, with no influence upon transplant outcomes (Table 28). Similar rates of relapse (41.9% versus 33.3%, p=0.933), OS (40.3% versus 44.4%, p=0.929), and EFS (46.7% versus 44.4%, p=0.800) were identified in KIR2DL1-R^{245} positive and negative donors, respectively.

6.3.2 Effect of Homozygosity and Heterozygosity of KIR2DL1 positions 114 and 245

Homozygosity and heterozygosity for the KIR2DL1 residue 114 motif offered variable influences upon post-HPCT outcomes (Figure 35). KIR2DL1-P/L^{114} heterozygous positive donor transplants presented intermediate probability of 3-year OS (47.6%), between that of KIR2DL1-P^{114} homozygous donor transplants (66.7%) and KIR2DL1-L^{114} homozygous positive donor transplants (29.0%) (p=0.030). Though clear separation in the 3-year OS Kaplan Meier curves was observed between the three characteristics, the HR of 3-year all-cause mortality for transplants using KIR2DL1-P/L^{114} heterozygous donors did not significantly differ from KIR2DL1-L^{114} homozygous donors (HR=0.67, 95% CI=0.32-1.37, p=0.271) or KIR2DL1-P^{114} homozygous donors (HR=1.80, 95% CI=0.76-4.24, p=0.180) (Table 29). KIR2DL1-P/L^{114} heterozygous donor transplants offered similar incidence of 3-year relapse (54.8%) to those of KIR2DL1-L^{114} homozygous donor transplants (52.3%), with KIR2DL1-P^{114} homozygous donor transplants presenting the lowest incidence (17.8%) (p=0.041, p_c=0.123). The 3-year EFS outcomes demonstrated an

intermediary between the former two findings, with KIR2DL1-P/L^{114} heterozygous donor transplants (33.3% 3-year EFS) presenting EFS closer to that of KIR2DL1-L^{114} homozygous donors (25.8%) than KIR2DL1-P^{114} homozygous donors (63.3%) (p=0.028, p_c=0.084). In all three post-transplant outcomes investigated, KIR2DL1 negative donors demonstrated similar outcomes to KIR2DL1-L^{114} homozygous donor transplants.

KIR2DL1 Amino Acid position	Category	n (%)	Overall Mortality HR (95% CI)	Event HR (95% CI)	Relapse HR (95% CI)
114	L hom.	31 (36.0%)	1 (p=0.040)	1 (p=0.065)	1 (p=0.039)
	P/L hetero.	21 (24.4%)	0.67 (0.32-1.37) (p=0.271)	0.82 (0.36-1.88) (p=0.646)	0.82 (0.42-1.59) (p=0.556)
	P hom.	30 (34.9%)	0.37 (0.18-0.79) (p=0.009)	0.27 (0.10-0.74) (p=0.011)	0.37 (0.18-0.76) (0=0.007)
	KIR2DL1 Neg,	4 (4.7%)	1.50 (0.45-5.03) (p=0.510)	1.36 (0.31-5.97) (p=0.681)	1.30 (0.39-4.35) (p=0.667)
245	R hom.	58 (67.4%)	1 (p=0.209)	1 (p=0.289)	1 (p=0.239)
	RC hetero.	19 (22.1%)	0.52 (0.23-1.18) (p=0.119)	0.59 (0.28-1.22) (p=0.154)	0.41 (0.14-1.18) (p=0.097)
	C hom.	5 (5.8%)	0.56 (0.14-2.34) (p=0.429)	0.49 (0.12-2.04) (p=0.329)	0.38 (0.5-2.84) (p=0.348)
	KIR2DL1 Neg,	4 (4.7%)	1.92 (0.59-6.28) (p=0.280)	1.61 (0.50-5.24) (p=0.428)	1.66 (0.39-7.04) (p=0.493)

Table 29 – Influence of KIR2DL1-P/L^{114} and -C/R^{245} homozygosity and heterozygosity upon post-transplant outcomes in T cell depleted RIC HPCT (Cohort B) (n=86).

Transplants using KIR2DL1-P^{114} homozygous donors presented reduced risk of mortality, events, and relapse compared to transplants using KIR2DL1-L^{114} homozygous donors. No other significant risk was observed.

Comparing KIR2DL1-P^{114} homozygous donor transplants and KIR2DL1-L^{114} homozygous donor transplants further re-enforced the benefits of KIR2DL1-P^{114} donors (Figure 35). Donors homozygous for KIR2DL1-P^{114} presented significantly improved 3-year EFS (63.3% versus 33.3%, p=0.044, p_c=0.088) and reduced relapse incidence (17.8% versus 52.3%, p=0.031, p_c=0.062) compared to transplants using KIR2DL1-L/P^{114} heterozygous donors.

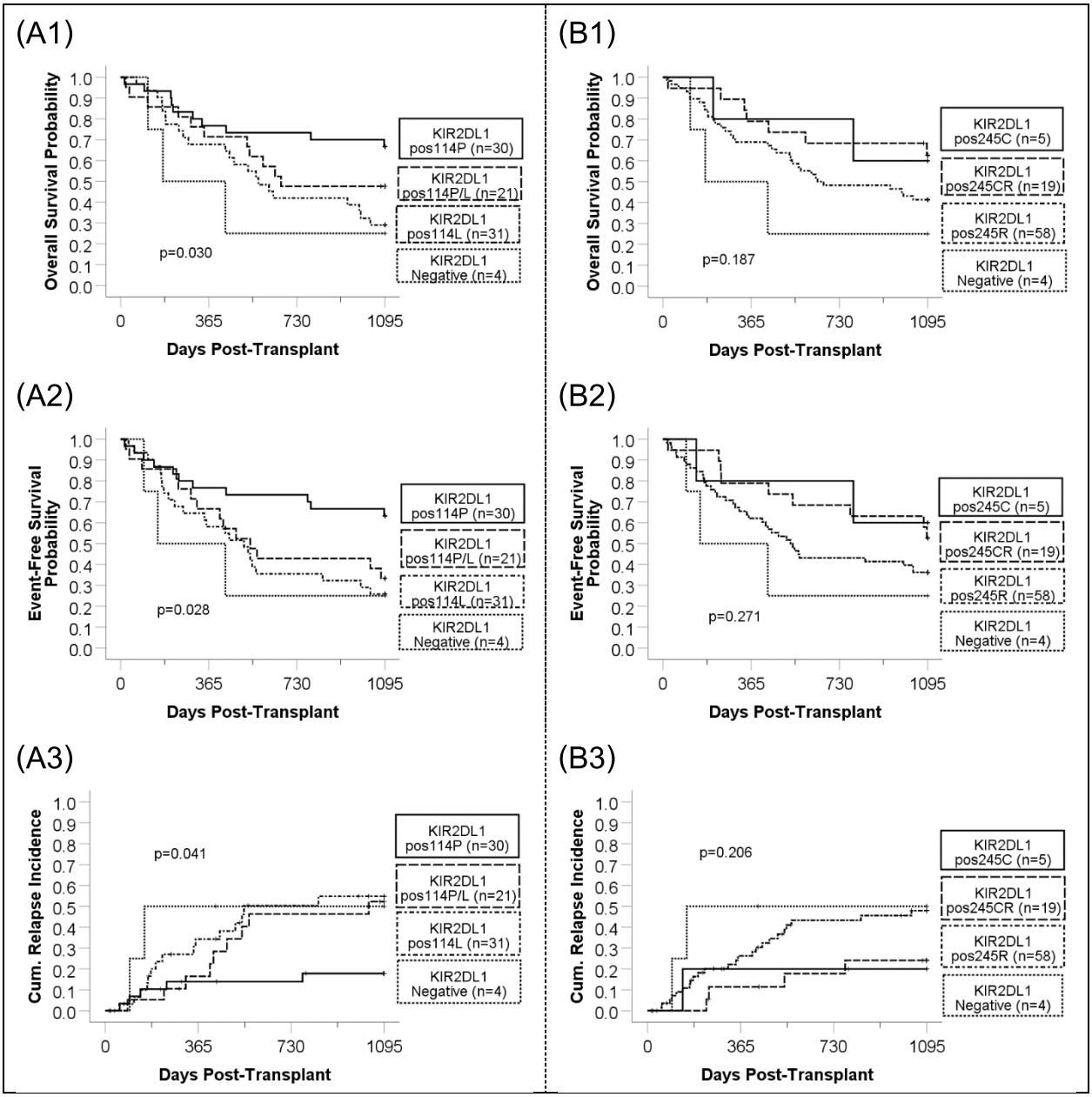


Figure 35 – Influence of KIR2DL1-P/L^{114} and -C/R^{245} homozygosity and heterozygosity upon post-transplant outcomes in T cell depleted RIC HPCT (Cohort B) (n=86).

KIR2DL1-P/L^{114} homozygosity and heterozygosity displayed significant spread in (A1) 3-year OS (p=0.030, p_c=0.090), (A2) EFS (p=0.028, p_c=0.084), and (A3) relapse incidence (p=0.041, p_c=0.123). Significance was not reach when assessing homozygosity/heterozygosity of KIR2DL1-C/R^{245} for (B1) 3-year OS (p=0.187), (B2) EFS (p=0.271), or (B3) relapse incidence (p=0.206).

KIR2DL1-C^{245} homozygous donors presented a trend towards superior HPCT outcomes, with KIR2DL1-C/R^{245} heterozygous donor transplants demonstrating similar outcomes to those of KIR2DL1-C^{245} homozygous donor transplants, though these findings did not reach significance (Figure 35, Table 29). KIR2DL1-C/R^{245}

heterozygous and -C^{245} homozygous donors presented optimal outcomes, with decreased incidence of 3-year relapse (20.0% and 24.1%, respectively, p=0.206), and improved 3-year OS (62.7% and 60.0%, p=0.187) and EFS (52.6% and 60.0%, p=0.271). KIR2DL1-R^{245} homozygous and KIR2DL1 negative donors presented inferior post-transplant outcomes in 3-year relapse incidence (48.0% and 50.0%, respectively, p=0.26), OS (41.4% and 25.0%, p=0.187) and EFS (36.2% and 25.0%, p=0.271).

6.3.3 Combining effects of KIR2DL1 positions 114 and 245

With the findings that KIR2DL1-P^{114} and -C^{245} positive donors improved post-transplant outcomes of relapse incidence, OS, and EFS, this raised questions of whether these two findings could be combined into a more nuanced model. The data was reassessed using the KIR2DL1-P/L^{114} homozygous/heterozygous stratification as the basis of the potential novel donor assessment model, with donors also positive for KIR2DL1-C^{245} marked as 'plus' (e.g. 'KIR2DL1-P^{114}+'). In accordance with the allele group SNP compositions identified in Chapter 5.3.1, 'plus' donor groups were identified for KIR2DL1-P^{114} homozygous and -P/L^{114} heterozygous donors, and no 'plus' donors were identified in the KIR2DL1-L^{114} homozygous donor group. This resulted in the following donor strata: 18.6% KIR2DL1-P^{114} homozygous plus (n=16), 16.2% KIR2DL1-P^{114} homozygous (n=14), 9.3% KIR2DL1- P/L^{114} heterozygous plus (n=8), 15.1% KIR2DL1-P/L^{114} heterozygous (n=13), 36.0% KIR2DL1-L^{114} homozygous (n=31), and 4.7% KIR2DL1 negative (n=4).

The 'plus' donors presented similar post-transplant outcomes to their respective standard grouping (Figure 36).

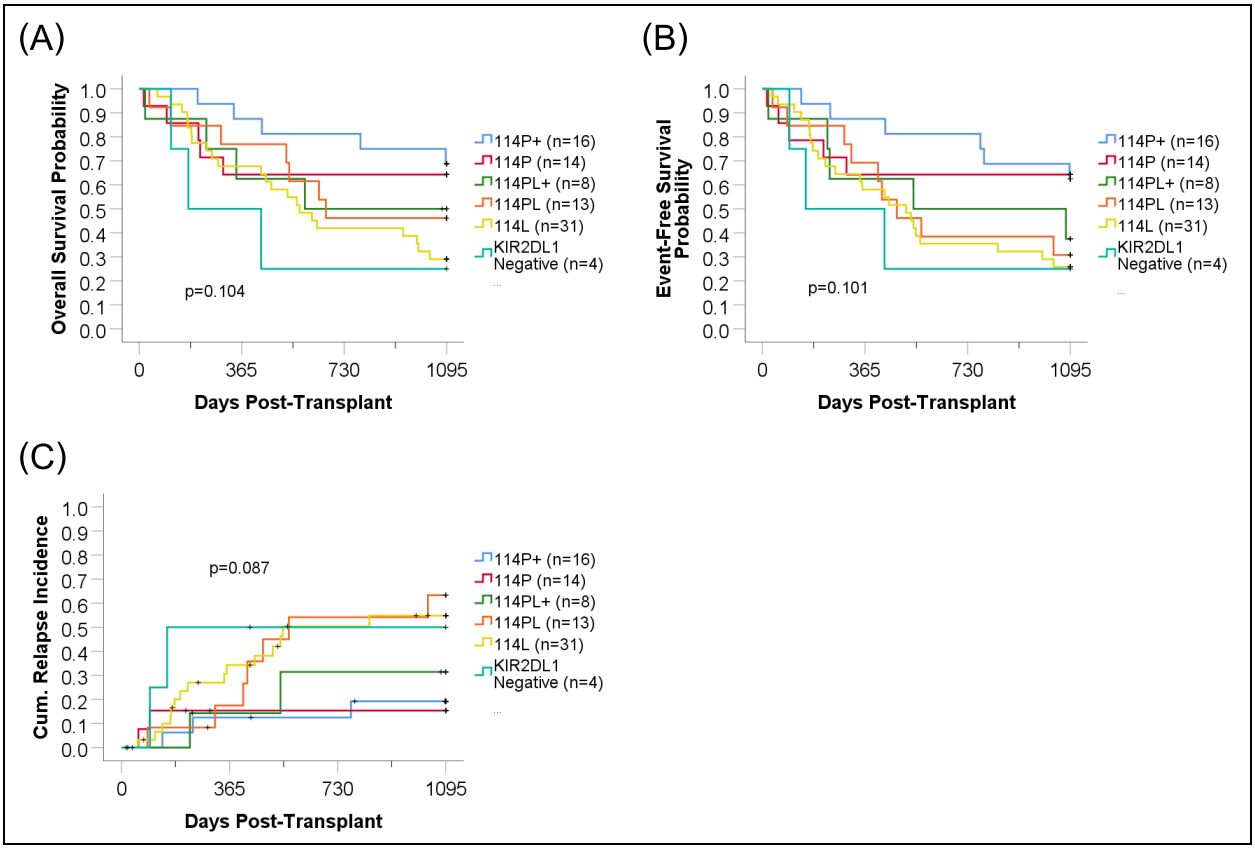


Figure 36 – Presentation of a potential donor assessment algorithm for KIR2DL1 polymorphism in T cell depleted RIC HPCT (Cohort B) (n=86)

(A) 3-year OS (p=0.104), (B) EFS (p=0.101), and (C) relapse incidence (p=0.087, p_c=0.174). The proposed algorithm assesses the donor for KIR2DL1-P/L^{114} homozygosity/heterozygosity. Donors possessing KIR2DL1-C^{245} provide additional weighting. The cohort size was insufficient to evaluate the value of this algorithm, necessitating further work with a larger cohort.

6.4 Discussion

This study has demonstrated that residue 114 of KIR2DL1 appears to present, or is linked to, the primary driving force in the beneficial transplantation outcomes observed in donor selection on the basis of KIR2DL1 allelic polymorphism. This SNP is dimorphic, with KIR2DL1 glycoproteins possessing either proline (P^{114}) or leucine (L^{114}) at this position. HPCT donors possessing KIR2DL1-P^{114} reduced the risk of 3-year relapse (HR=0.46, 95% CI=0.23-0.94, p=0.032, p_c=0.096), with associated reduction in all-cause mortality (HR=0.46, 95% CI=0.26-0.83, p=0.010, p_c=0.040) and experiencing events (HR=0.52, 95% CI=0.30-0.90, p=0.020, p_c=0.068). Conversely, KIR2DL1-L^{114} positive donors correlated with increased risk of relapse (HR=2.65,

95% CI=1.14-6.17, p=0.024, p_c=0.096), all-cause mortality (HR=1.91, 95% CI=1.00-3.63, p=0.050, p_c=0.150), and experiencing events (HR=2.12, 95% CI=1.14-3.94, p=0.017, p_c=0.068).

The negative effects of KIR2DL1-L^{114} appear to overpower the benefits of KIR2DL1-P^{114}, with donors heterozygous for KIR2DL1 alleles with this amino acid motif presenting similar rates of relapse and EFS to donors homozygous for KIR2DL1-L^{114}. In contrast, KIR2DL1-P/L^{114} heterozygous donors did present an intermediary rate of OS, with 47.6% 3-year OS compared to 66.7% in KIR-2DL1-P^{114} homozygous donors and 29.0% in KIR2DL1-L^{114} homozygous donors (p=0.030, p_c=0.090). This suggests that the interactions between KIR2DL1-P^{114} and -L^{114} receptors are likely to be situational, with KIR2DL1-P^{114} receptors compensating for KIR2DL1-L^{114} receptors in some scenarios, but not in others. Further work is required to interrogate these situation-dependent interactions in detail.

The dimorphic position at residue 245, with arginine (R^{245}) or cysteine (C^{245}) played a lesser role in improving transplant outcomes. Donors possessing KIR2DL1-C^{245} trended towards reducing risk of 3-year relapse (HR=0.39, 95% CI=0.15-1.02, p=0.055, p_c=0.110), with associated decreases to all-cause mortality (p=0.51, 95% CI=0.25-1.06, p=0.072, p_c=0.150) and experiencing events (HR=0.55, 95% CI=0.283-1.08, p=0.082, p_c=0.164), but R^{245} is only ever found in association with P^{114}. KIR2DL1-R^{245} appeared to be neutral, with donors possessing KIR2DL1-R^{245} presenting similar transplant outcomes compared to KIR2DL1-R^{245} negative donors.

Residue 114 is located within Exon 4 of KIR2DL1, encoding the extracellular D2 domain, and has been demonstrated to play an instrumental role in the binding avidity of the receptor. KIR2DL1-P^{114} receptors present increased binding avidity compared

to KIR2DL1-L^{114} receptors. (Hilton et al., 2015a). Residue 245 is located within exon 7 of KIR2DL1, encoding the transmembrane region, and has been shown to significantly influence the signalling capacity of the receptor. KIR2DL1-C^{245} receptors display significantly reduced signalling capacity compared to KIR2DL1-R^{245} (Bari et al., 2011; Hilton et al., 2015a; Le Luduec et al., 2019). As a consequence of reduced signalling, KIR2DL1-C^{245} positive NK cells exhibit weaker functional capacity, with reduced degranulation (Le Luduec et al., 2019).

The immunological basis for the variance in post-HPCT outcomes in the context of these two sub-types of KIR2DL1 receptors is unknown. KIR genes are expressed in a stochastic variegated manner, with copy number variation (CNV) primarily influencing KIR expression levels on the population level, rather than level of the individual cell (i.e. high CNV results in more NK cells expressing the gene, but does not lead to a single NK cell expressing higher levels of the gene) (Santourlidis et al., 2002; Béziat et al., 2013; Manser et al., 2015; Le Luduec et al., 2019). Consequently, individuals who are KIR2DL1-P/L^{114} heterozygous may produce different proportions of NK cell sub-populations expressing the KIR2DL1-P^{114} receptor compared to individuals homozygous for this residue motif.

On the level of KIR gene content (gene presence/absence), the NK cell sub-population composition has substantial effects on the wider NK cell immune response, and so is likely to influence the NK cell response to malignancy and infection (Manser et al., 2015). The NK cell sub-population composition respective to signature SNP motifs may exhibit similar effects upon immune responses, with KIR2DL1-P/L^{114} heterozygous individuals possessing different proportion of KIR2DL1-P^{114} positive NK cells compared to individuals homozygous for KIR2DL1-P^{114}. Consequently,

these NK cell sub-populations take different lengths of time to reach therapeutic levels. This may explain why the advantageous effects of the KIR2DL1-P^{114} allele group is able to compensate partially for the detrimental effects of KIR2DL1-L^{114} in the case of OS, but is entirely negated in relapse incidence. Further work is required to explore how homozygosity and heterozygosity for allelic polymorphism influences the composition and immune response of NK cell sub-populations.

When considering the construction of a novel model for HPCT donor selection protocols, a number of factors must be considered: (i) financial cost of testing, (ii) workload for testing, (iii) the proportion of optimal donors within the international donor pool, (iv) the number of hierarchical tranches proposed by the model, and (v) the number of donors that require testing before identifying the optimal donor. Financial cost and workload for testing place pressure upon the transplantation centre laboratories, requiring sufficient benefits to HPCT outcomes to justify this expense.

Assessing for presence of KIR2DL1-L/P^{114} allele groups in prospective HPCT donors as part of a donor selection algorithm satisfies a majority of these considerations, with a clear hierarchy of donors and approximately one in three (35%) donors presenting the optimal characteristic of KIR2DL1-P^{114} homozygous. Testing for signature SNP motifs can be achieved relatively inexpensively with high throughput and minimal workload using real-time PCR or sequence-specific primer (PCR-SSP) technology, negating the requirements for costly full gene sequencing. Other groups have developed similar kits to define key polymorphisms of KIR genes, with one PCR-SSP test protocol capable of discerning between major groups of KIR2DL1 with a minimum of six PCR test wells per sample (Boudreau et al., 2014; Le Luduec et al., 2018).

The cohort size of this single centre presented a major limitation for the scope of this study, with insufficient statistical power to fully assess the stratification donors in respect to their KIR2DL1 genetic characteristics. Of specific interest for a larger multi-centre cohort would be the more detailed comparison of transplants using donors homozygous versus heterozygous for each KIR2DL1 characteristic, and the investigation into whether the additional assessment for KIR2DL1-C^{245} potentially provides further finesse to the proposed donor selection clinical model. A larger cohort could also facilitate assessment of a more complex algorithm, such as the combined assessment of KIR2DL1 and KIR3DL1 allelic polymorphism to interrogate the interactions between centromeric and telomeric KIR genes (Boudreau et al., 2017).

Despite these limitations in statistical power, the data presented in this study offers an initial proof of principle that this area of KIR genomics is of merit for investigation in a larger multi-centre cohort study. This study raises many further questions regarding the variance observed in KIR2DL1-P/L^{114} homo/heterozygosity in HPCT donors, and whether KIR2DL1-C^{245} provides additional significance to a clinical model based around donor selection on the basis of KIR2DL1-P/L^{114}.

Chapter 6 References

Bari, R., Leung, M., Turner, V. E., Embrey, C., Rooney, B., Holladay, M. & Leung, W. (2011) Molecular determinant-based typing of KIR alleles and KIR ligands. *Clin Immunol,* 138(3), 274-81.

Béziat, V., Traherne, J. A., Liu, L. L., Jayaraman, J., Enqvist, M., Larsson, S., Trowsdale, J. & Malmberg, K. J. (2013) Influence of KIR gene copy number on natural killer cell education. *Blood,* 121(23), 4703-7.

Boudreau, J. E., Giglio, F., Gooley, T. A., Stevenson, P. A., Le Luduec, J. B., Shaffer, B. C., Rajalingam, R., Hou, L., Hurley, C. K., Noreen, H., Reed, E. F., Yu, N., Vierra-Green, C., Haagenson, M., Malkki, M., Petersdorf, E. W., Spellman, S. & Hsu, K. C. (2017) KIR3DL1/HLA-B Subtypes Govern Acute Myelogenous Leukemia Relapse After Hematopoietic Cell Transplantation. *J Clin Oncol,* 35(20), 2268-2278.

Boudreau, J. E., Le Luduec, J. B. & Hsu, K. C. (2014) Development of a novel multiplex PCR assay to detect functional subtypes of KIR3DL1 alleles. *PLoS One,* 9(6), e99543.

Broder, M. S., Quock, T. P., Chang, E., Reddy, S. R., Agarwal-Hashmi, R., Arai, S. & Villa, K. F. (2017) The cost of hematopoietic stem-cell transplantation in the United States. *Am Health Drug Benefits,* 10(7), 366-374.

Fleischhauer, K., Fernandez-Vina, M. A., Wang, T., Haagenson, M., Battiwalla, M., Baxter-Lowe, L. A., Ciceri, F., Dehn, J., Gajewski, J., Hale, G. A., Heemskerk, M. B., Marino, S. R., McCarthy, P. L., Miklos, D., Oudshoorn, M., Pollack, M. S., Reddy, V., Senitzer, D., Shaw, B. E., Waller, E. K., Lee, S. J. & Spellman, S. R. (2014) Risk associations between HLA-DPB1 T-cell epitope matching and outcome of unrelated hematopoietic cell transplantation are independent of HLA-DPA1. *Bone Marrow Transplant,* 49(9), 1176-83.

Gaetano, J. (2018) *Holm-Bonferroni sequential correction: An Excel calculator (1.3) [Microsoft Excel Workbook]* [Online]. Available: https://www.researchgate.net/publication/322568540_Holm-Bonferroni_sequential_correction_An_Excel_calculator_13 [Accessed].

Hilton, H. G., Guethlein, L. A., Goyos, A., Nemat-Gorgani, N., Bushnell, D. A., Norman, P. J. & Parham, P. (2015a) Polymorphic HLA-C receptors balance the functional characteristics of KIR haplotypes. *J Immunol,* 195(7), 3160-70.

Khera, N., Zeliadt, S. B. & Lee, S. J. (2012) Economics of hematopoietic cell transplantation. *Blood,* 120(8), 1545-51.

Le Luduec, J. B., Boudreau, J. E., Freiberg, J. C. & Hsu, K. C. (2019) Novel Approach to Cell Surface Discrimination Between KIR2DL1 Subtypes and KIR2DS1 Identifies Hierarchies in NK Repertoire, Education, and Tolerance. *Front Immunol,* 10, 734.

Le Luduec, J. B., Kudva, A., Boudreau, J. E. & Hsu, K. C. (2018) Novel multiplex PCR-SSP method for centromeric KIR allele discrimination. *Sci Rep,* 8(1), 14853.

Manser, A. R., Weinhold, S. & Uhrberg, M. (2015) Human KIR repertoires: shaped by genetic diversity and evolution. *Immunol Rev,* 267(1), 178-96.

Mayor, N. P., Hayhurst, J. D., Turner, T. R., Szydlo, R. M., Shaw, B. E., Bultitude, W. P., Sayno, J. R., Tavarozzi, F., Latham, K., Anthias, C., Robinson, J., Braund, H., Danby, R., Perry, J., Wilson, M. C., Bloor, A. J., McQuaker, I. G., MacKinnon, S., Marks, D. I., Pagliuca, A., Potter, M. N., Potter, V. T., Russell,

N. H., Thomson, K. J., Madrigal, J. A. & Marsh, S. G. E. (2019) Recipients Receiving Better HLA-Matched Hematopoietic Cell Transplantation Grafts, Uncovered by a Novel HLA Typing Method, Have Superior Survival: A Retrospective Study. *Biol Blood Marrow Transplant,* 25(3)**,** 443-450.

Petersdorf, E. W., Anasetti, C., Martin, P. J., Gooley, T., Radich, J., Malkki, M., Woolfrey, A., Smith, A., Mickelson, E. & Hansen, J. A. (2004) Limits of HLA mismatching in unrelated hematopoietic cell transplantation. *Blood,* 104(9)**,** 2976-80.

Santourlidis, S., Trompeter, H. I., Weinhold, S., Eisermann, B., Meyer, K. L., Wernet, P. & Uhrberg, M. (2002) Crucial role of DNA methylation in determination of clonally distributed killer cell Ig-like receptor expression patterns in NK cells. *The Journal of Immunology,* 169(8)**,** 4253-61.

Shaw, B. E., Logan, B. R., Spellman, S. R., Marsh, S. G. E., Robinson, J., Pidala, J., Hurley, C., Barker, J., Maiers, M., Dehn, J., Wang, H., Haagenson, M., Porter, D., Petersdorf, E. W., Woolfrey, A., Horowitz, M. M., Verneris, M., Hsu, K. C., Fleischhauer, K. & Lee, S. J. (2018) Development of an Unrelated Donor Selection Score Predictive of Survival after HCT: Donor Age Matters Most. *Biol Blood Marrow Transplant,* 24(5)**,** 1049-1056.

Shaw, B. E., Mayor, N. P., Szydlo, R. M., Bultitude, W. P., Anthias, C., Kirkland, K., Perry, J., Clark, A., Mackinnon, S., Marks, D. I., Pagliuca, A., Potter, M. N., Russell, N. H., Thomson, K., Madrigal, J. A. & Marsh, S. G. E. (2017) Recipient/donor HLA and CMV matching in recipients of T-cell-depleted unrelated donor haematopoietic cell transplants. *Bone Marrow Transplant,* 52(5)**,** 717-725.

Chapter 7 | Concluding Remarks

The findings described in this thesis have expanded upon existing knowledge of KIR population frequencies and the implementation of KIR allelic polymorphism within HPCT algorithms. Combined, the four studies have investigated the KIR population genomics in a local transplant cohort, compared the effectiveness of existing KIR assessment algorithms in HPCT, and investigated the influence of KIR allele groups in HPCT outcomes to potentially contribute towards a novel clinical algorithm. Finally, re-assessment of KIR allele groups in HPCT outcomes as single nucleotide polymorphisms (SNPs) has been completed as a means of reducing the cost of testing for the transplant centre.

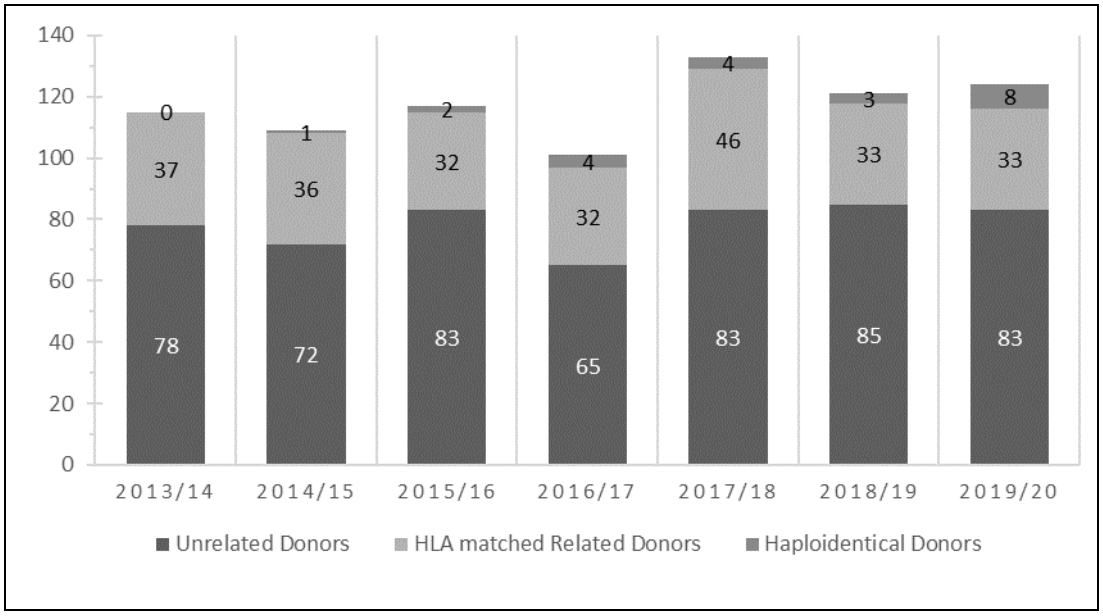


Figure 37 — Number of HPCT transplants carried out in Manchester, 2013/14 to 2019/20.
Based on local clinical audit data.

The Manchester University NHS Foundation Trust is one of the busiest transplant centres in the United Kingdom, with over one hundred HPCT operations carried out per annum (Figure 37). The high cost of HPCT – among the most expensive treatments carried out in medicine – and the poor prognosis of many haematological malignancies post-transplant (even with optimal treatment options) means that any

incremental improvement in post-transplant outcomes is of great benefit to both for patient care and the economics of transplant centres (Khera et al., 2012; Broder et al., 2017; Shaw et al., 2017). Improvements in post-transplant outcomes leads to a decline in patient mortality and morbidity rates, and an associated reduction in the requirement for re-transplantation and other costly treatments to manage disease progression.

The field of KIR genomics is rapidly expanding, with recent findings of recombinant KIR genes, novel KIR haplotype arrangements, and identification of novel KIR alleles (Roe et al., 2017; Misra et al., 2018; Falco et al., 2019). The fast pace of these new findings, in combination with new technologies such as NGS, may soon lead to an understanding of KIR that matches the in-depth knowledge of HLA. In addition, it is now realised that the interactions between NK cells and T lymphocytes are major factors in the control of viral infection and malignancy, presenting an exciting frontier of H&I that is yet to be exploited in clinical transplantation (Boelen et al., 2018).

Cross-field collaboration is now imperative to maximise development of this area, particularly between immunogeneticists and molecular biologists to better understand the link between KIR genomics and proteomics. The establishment of matrices for high/low expression levels and high/low binding affinity of KIR allele gene products derived from molecular studies would be an effective tool for the H&I community to apply to transplantation.

The lack of a universally accepted HPCT model for the application of KIR genetics is concerning. It is possible that no model can be applied universally due to the diversity of KIR genetics between different populations, relying instead upon broad characteristics acting as a proxy for a more refined genetic feature – an association that may not be present in all populations. By assessing allelic polymorphism of KIR

genes, more universally applicable improvements in transplant outcomes may follow. Historic improvements to HLA matching protocols have demonstrated such a trajectory in progression of clinical models alongside the development of basic scientific technique and technology (Fleischhauer et al., 1990; Santamaria et al., 1994; Petersdorf et al., 1998; Petersdorf et al., 2004; Shaw et al., 2017; Mayor et al., 2019). The greatest contrast between the developments in HLA and KIR has been in how HLA assessment algorithms were dependent upon waiting for improvement in scientific technology to facilitate research and implementation, while current research into KIR genetics has accessed a wide array of modern technologies to develop understanding at a far more rapid rate of progress.

The data presented within this body of work contribute to the mounting evidence that the assessment of KIR genomics in clinical algorithms is worthwhile. Translating sequencing evidence into SNPs provides a compromise in testing to accommodate the requirements of clinical laboratories while not compromising on the quality of information required for effective patient care. Testing for KIR2DL1-P^{114} via SSP or qPCR is a cheap test that could be routinely implemented by all clinical laboratory in the UK. Recent evidence that the transplant conditioning regime interacts with the influence of KIR is one step further towards understanding the principles of KIR in transplantation (Falco et al., 2019). Moving forward, it will be of interest to examine how the findings presented in this series of studies in a single centre compare to that of other centre cohorts.

A further hurdle in donor KIR genotyping is in the logistics of who should be responsible for carrying out the testing. Donor genetic information provided by the donor registry at the time of registration and allows clinical laboratories to screen for

optimal donors before they order the donor samples and incur financial costs. Without this pre-screen information, transplant centres may need to order more donor samples to increase the likelihood of identifying an optimal donor. For example, at least three donors would likely need to be tested to identify one KIR2DL1-P^{114} homozygous donor. It is good clinical practice for a reserve donor to be identified in case the first donor is unavailable or unsuitable, and so the requirement to potentially order at least five donors to identify two optimal donors is likely to be unrealistic for many transplant centres to be used in all patients.

Some donor registries are committing to KIR genotyping at registration, with the German registry pledging to sequence millions of registered donors (2.8 million at the beginning of 2019) with the explicit aim of accelerating the potential timeline from ordering donors to transplantation (Weisdorf et al., 2019; Schmidt et al., 2020; Solloch et al., 2020). Other registries are more cautious in carrying out additional testing due to the costs involved, which must ultimately be passed on to the transplant centres as the service users. Consequently, providing convincing evidence for the benefits of KIR genotyping is essential to encourage more donor registries to carry out this testing.

The value of a clinical tool for the means of HPCT donor selection is predicated on the clinical benefits the test provides for patient care versus the financial and workload cost to carry out the test. Over the past ten years, a number of criteria have been demonstrated to provide a large clinical benefit including HLA matching of HLA-A, B, C, DRB1, and DQB1 between the recipient and donor to the highest possible resolution, HLA-DPB1 compatibility assessments, CMV serostatus matching between recipient and donor, and selecting donors under 30 years (Crocchiolo et al., 2009;

Fleischhauer et al., 2014; Shaw et al., 2017; Shaw et al., 2018; Mayor et al., 2019). These criteria have the benefit of being either an integral part of the established transplant work-up process (e.g. HLA genotyping and CMV testing) or a simple metric that can be applied with no additional cost (e.g. selecting donors on the basis of age).

The number of different criteria for donor selection highlight one of the greatest concerns for KIR genotyping algorithms: can KIR genotyping algorithms provide a means of improving post-transplant outcomes in otherwise inferior transplants, such as HLA mismatched transplants? Owing to the nature of the wide polymorphic diversity in both KIR and HLA genomics, this would necessitate a large national multi-centre study beyond the scope of this thesis. As more significant factors of transplantation outcomes are identified, it is essential that the clinical field establishes a hierarchy of compatibility that allows transplant centres to identify the optimal donor in a range of transplant settings and situations, including in HLA incompatible transplantation. The latter is key for equity of access in HPCT, particularly in aiding minority ethnic populations currently under-served by donor registries (Anonymous, 2020a; Anonymous, 2020b; Neujahr et al., 2020).

The potential for introducing KIR genotyping as a routine test must consider barriers to entry on a number of fronts. Firstly, KIR testing is an additional test, with further financial burden and workload required to provide this data. Secondly, transplant centres are often unable to identify optimal donors for some patients using the current established criteria, and so any additional criteria are perceived to be superfluous to requirement. Finally, the reported benefits are not considered to be sufficient to justify the cost involved in testing. Consequently, few transplant centres in the United

Kingdom are currently willing to consider implementing KIR genotyping on the basis of the available published data.

To conclude, the data and results presented here-in are encouraging for the future of KIR research and the H&I field. Irrespective of the impact these have upon the future of transplantation algorithms, they provide a valuable contribution to the continuing debate over developing better assessment metrics for improving patient outcomes.

Chapter 7 References

Anonymous. (2020a) *Anthony Nolan state of the registry 2019-2020* [Online]. Available: https://www.anthonynolan.org/sites/default/files/2266ST_StateOfTheRegistry_1920_v6.pdf [Accessed 31/08/2020 2020].

Anonymous. (2020b) *Be the Match: 2019 Report to the Community* [Online]. Available: https://bethematch.org/workarea/downloadasset.aspx?id=15032386599 [Accessed 31/08/2020 2020].

Boelen, L., Debebe, B., Silveira, M., Salam, A., Makinde, J., Roberts, C. H., Wang, E. C. Y., Frater, J., Gilmour, J., Twigger, K., Ladell, K., Miners, K. L., Jayaraman, J., Traherne, J. A., Price, D. A., Qi, Y., Martin, M. P., Macallan, D. C., Thio, C. L., Astemborski, J., Kirk, G., Donfield, S. M., Buchbinder, S., Khakoo, S. I., Goedert, J. J., Trowsdale, J., Carrington, M., Kollnberger, S. & Asquith, B. (2018) Inhibitory killer cell immunoglobulin-like receptors strengthen CD8(+) T cell-mediated control of HIV-1, HCV, and HTLV-1. *Sci Immunol,* 3(29)**,** eaao2892.

Broder, M. S., Quock, T. P., Chang, E., Reddy, S. R., Agarwal-Hashmi, R., Arai, S. & Villa, K. F. (2017) The cost of hematopoietic stem-cell transplantation in the United States. *Am Health Drug Benefits,* 10(7)**,** 366-374.

Crocchiolo, R., Zino, E., Vago, L., Oneto, R., Bruno, B., Pollichieni, S., Sacchi, N., Sormani, M. P., Marcon, J., Lamparelli, T., Fanin, R., Garbarino, L., Miotti, V., Bandini, G., Bosi, A., Ciceri, F., Bacigalupo, A., Fleischhauer, K., Gruppo Italiano Trapianto di Midollo Osseo, C. S. E. e. T. C. & Italian Bone Marrow Donor, R. (2009) Nonpermissive HLA-DPB1 disparity is a significant independent risk factor for mortality after unrelated hematopoietic stem cell transplantation. *Blood,* 114(7)**,** 1437-44.

Falco, M., Sivori, S., Meazza, R. & Pende, D. (2019) Report from the Eleventh Killer Immunoglobulin-like Receptor (KIR) Workshop: Novel insights on KIR polymorphism, ligand recognition, expression and function. *HLA,* 94(2)**,** 100-110.

Fleischhauer, K., Fernandez-Vina, M. A., Wang, T., Haagenson, M., Battiwalla, M., Baxter-Lowe, L. A., Ciceri, F., Dehn, J., Gajewski, J., Hale, G. A., Heemskerk, M. B., Marino, S. R., McCarthy, P. L., Miklos, D., Oudshoorn, M., Pollack, M. S., Reddy, V., Senitzer, D., Shaw, B. E., Waller, E. K., Lee, S. J. & Spellman, S. R. (2014) Risk associations between HLA-DPB1 T-cell epitope matching and outcome of unrelated hematopoietic cell transplantation are independent of HLA-DPA1. *Bone Marrow Transplant,* 49(9)**,** 1176-83.

Fleischhauer, K., Kernan, N. A., O'Reilly, R. J., Dupont, B. & Yang, S. Y. (1990) Bone Marrow–Allograft Rejection by T Lymphocytes Recognizing a Single Amino Acid Difference in HLA-B44. *New England Journal of Medicine,* 323(26)**,** 1818-1822.

Khera, N., Zeliadt, S. B. & Lee, S. J. (2012) Economics of hematopoietic cell transplantation. *Blood,* 120(8)**,** 1545-51.

Mayor, N. P., Hayhurst, J. D., Turner, T. R., Szydlo, R. M., Shaw, B. E., Bultitude, W. P., Sayno, J. R., Tavarozzi, F., Latham, K., Anthias, C., Robinson, J., Braund, H., Danby, R., Perry, J., Wilson, M. C., Bloor, A. J., McQuaker, I. G., MacKinnon, S., Marks, D. I., Pagliuca, A., Potter, M. N., Potter, V. T., Russell,

N. H., Thomson, K. J., Madrigal, J. A. & Marsh, S. G. E. (2019) Recipients Receiving Better HLA-Matched Hematopoietic Cell Transplantation Grafts, Uncovered by a Novel HLA Typing Method, Have Superior Survival: A Retrospective Study. *Biol Blood Marrow Transplant,* 25(3)**,** 443-450.

Misra, M. K., Augusto, D. G., Martin, G. M., Nemat-Gorgani, N., Sauter, J., Hofmann, J. A., Traherne, J. A., Gonzalez-Quezada, B., Gorodezky, C., Bultitude, W. P., Marin, W., Vierra-Green, C., Anderson, K. M., Balas, A., Caro-Oleas, J. L., Cisneros, E., Colucci, F., Dandekar, R., Elfishawi, S. M., Fernandez-Vina, M. A., Fouda, M., Gonzalez-Fernandez, R., Grosse, A., Herrero-Mata, M. J., Hollenbach, S. Q., Marsh, S. G. E., Mentzer, A., Middleton, D., Moffett, A., Moreno-Hidalgo, M. A., Mossallam, G. I., Nakimuli, A., Oksenberg, J. R., Oppenheimer, S. J., Parham, P., Petzl-Erler, M. L., Planelles, D., Sanchez-Garcia, F., Sanchez-Gordo, F., Schmidt, A. H., Trowsdale, J., Vargas, L. B., Vicario, J. L., Vilches, C., Norman, P. J. & Hollenbach, J. A. (2018) Report from the killer-cell immunoglobulin-like receptors (KIR) component of the 17th International HLA and Immunogenetics Workshop. *Hum Immunol,* 79(12)**,** 825-33.

Neujahr, E., Schmidt, A. H. & Geist, S. (2020) DKMS Annual Report 2018.

Petersdorf, E. W., Anasetti, C., Martin, P. J., Gooley, T., Radich, J., Malkki, M., Woolfrey, A., Smith, A., Mickelson, E. & Hansen, J. A. (2004) Limits of HLA mismatching in unrelated hematopoietic cell transplantation. *Blood,* 104(9)**,** 2976-80.

Petersdorf, E. W., Gooley, T. A., Anasetti, C., Martin, P. J., Smith, A. G., Mickelson, E. M., Woolfrey, A. E. & Hansen, J. A. (1998) Optimizing Outcome After Unrelated Marrow Transplantation by Comprehensive Matching of HLA Class I and II Alleles in the Donor and Recipient. *Blood,* 92(10)**,** 3515-3520.

Roe, D., Vierra-Green, C., Pyo, C. W., Eng, K., Hall, R., Kuang, R., Spellman, S., Ranade, S., Geraghty, D. E. & Maiers, M. (2017) Revealing complete complex KIR haplotypes phased by long-read sequencing technology. *Genes Immun,* 18(3)**,** 127-34.

Santamaria, P., Reinsmoen, N. L., Lindstrom, A. L., Boyce-Jacino, M. T., Barbosa, J. J., Faras, A. J., McGlave, P. B. & Rich, S. S. (1994) Frequent HLA class I and DP sequence mismatches in serologically (HLA- A, HLA-B, HLA-DR) and molecularly (HLA-DRB1, HLA-DQA1, HLA-DQB1) HLA- identical unrelated bone marrow transplant pairs. *Blood,* 83(12)**,** 3834-3834.

Schmidt, A. H., Sauter, J., Baier, D. M., Daiss, J., Keller, A., Klussmeier, A., Mengling, T., Rall, G., Riethmuller, T., Schofl, G., Solloch, U. V., Torosian, T., Means, D., Kelly, H., Jagannathan, L., Paul, P., Giani, A. S., Hildebrand, S., Schumacher, S., Markert, J., Fussel, M., Hofmann, J. A., Schafer, T., Pingel, J., Lange, V. & Schetelig, J. (2020) Immunogenetics in stem cell donor registry work: The DKMS example (Part 2). *Int J Immunogenet,* 47(2)**,** 139-148.

Shaw, B. E., Logan, B. R., Spellman, S. R., Marsh, S. G. E., Robinson, J., Pidala, J., Hurley, C., Barker, J., Maiers, M., Dehn, J., Wang, H., Haagenson, M., Porter, D., Petersdorf, E. W., Woolfrey, A., Horowitz, M. M., Verneris, M., Hsu, K. C., Fleischhauer, K. & Lee, S. J. (2018) Development of an Unrelated Donor Selection Score Predictive of Survival after HCT: Donor Age Matters Most. *Biol Blood Marrow Transplant,* 24(5)**,** 1049-1056.

Shaw, B. E., Mayor, N. P., Szydlo, R. M., Bultitude, W. P., Anthias, C., Kirkland, K., Perry, J., Clark, A., Mackinnon, S., Marks, D. I., Pagliuca, A., Potter, M. N.,

Russell, N. H., Thomson, K., Madrigal, J. A. & Marsh, S. G. E. (2017) Recipient/donor HLA and CMV matching in recipients of T-cell-depleted unrelated donor haematopoietic cell transplants. *Bone Marrow Transplant,* 52(5), 717-725.

Solloch, U. V., Schefzyk, D., Schafer, G., Massalski, C., Kohler, M., Pruschke, J., Heidl, A., Schetelig, J., Schmidt, A. H., Lange, V. & Sauter, J. (2020) Estimation of German KIR Allele Group Haplotype Frequencies. *Front Immunol,* 11, 429.

Weisdorf, D., Cooley, S., Wang, T., Trachtenberg, E., Haagenson, M. D., Vierra-Green, C., Spellman, S., Spahn, A., Vogel, J., Kobusingye, H., Fehninger, T., Woolfrey, A., Devine, S., Ross, M., Waller, E. K., Sobecks, R., Parham, P., Guethlein, L. A., Marsh, S. G. E., Miller, J. & participating center writing, c. (2019) KIR donor selection: Feasibility in identifying better donors. *Biol Blood Marrow Transplant,* 25(1), e28-32.

Thesis Bibliography

Alicata, C., Pende, D., Meazza, R., Canevali, P., Loiacono, F., Bertaina, A., Locatelli, F., Nemat-Gorgani, N., Guethlein, L. A., Parham, P., Moretta, L., Moretta, A., Bottino, C., Norman, P. J. & Falco, M. (2016) Hematopoietic stem cell transplantation: Improving alloreactive Bw4 donor selection by genotyping codon 86 of KIR3DL1/S1. *Eur J Immunol,* 46(6)**,** 1511-7.

Anfossi, N., Andre, P., Guia, S., Falk, C. S., Roetynck, S., Stewart, C. A., Breso, V., Frassati, C., Reviron, D., Middleton, D., Romagne, F., Ugolini, S. & Vivier, E. (2006) Human NK cell education by inhibitory receptors for MHC class I. *Immunity,* 25(2)**,** 331-42.

Anonymous. (2011) Quality scores for next-generation sequencing (illumina).

Anonymous. (2014) *Immunogenomic Next Generation Sequencing Data Consortium.* [Online]. IDAWG. Available: http://igdawg.org/ngs.html [Accessed 16/05/2019].

Anonymous. (2017a) GenDx NGSgo Workflow: Compatible with Illumina. Instructions for use. Edition 4 2017/06.

Anonymous. (2017b) TruSight HLA v2 sequencing panel reference guide (Illumina) v1.

Anonymous. (2018a) GenDx KIR amplification edition 0.1, 2018/06 [PROTOTYPE].

Anonymous. (2018b) LABType SSO typing tests: Product insert Rev 29.

Anonymous. (2020a) *Anthony Nolan state of the registry 2019-2020* [Online]. Available: https://www.anthonynolan.org/sites/default/files/2266ST_StateOfTheRegistry_1920_v6.pdf [Accessed 31/08/2020 2020].

Anonymous. (2020b) *Be the Match: 2019 Report to the Community* [Online]. Available: https://bethematch.org/workarea/downloadasset.aspx?id=15032386599 [Accessed 31/08/2020 2020].

Ardui, S., Ameur, A., Vermeesch, J. R. & Hestand, M. S. (2018) Single molecule real-time (SMRT) sequencing comes of age: applications and utilities for medical diagnostics. *Nucleic Acids Res,* 46(5)**,** 2159-68.

Arima, N., Kanda, J., Tanaka, J., Yabe, T., Morishima, Y., Kim, S. W., Najima, Y., Ozawa, Y., Eto, T., Kanamori, H., Mori, T., Kobayashi, N., Kondo, T., Nakamae, H., Uchida, N., Inoue, M., Fukuda, T., Ichinohe, T., Atsuta, Y. & Kanda, Y. (2018) Homozygous HLA-C1 is associated with reduced risk of relapse after HLA-matched transplantation in patients with myeloid leukemia. *Biol Blood Marrow Transplant,* 24(4)**,** 717-25.

Artavanis-Tsakonas, K., Eleme, K., McQueen, K. L., Cheng, N. W., Parham, P., Davis, D. M. & Riley, E. M. (2003) Activation of a subset of human NK cells upon contact with Plasmodium falciparum-infected erythrocytes. *J Immunol,* 171(10)**,** 5396-405.

Augusto, D. G. (2016) The Impact of KIR Polymorphism on the Risk of Developing Cancer: Not as Strong as Imagined? *Front Genet,* 7**,** 121.

Augusto, D. G. & Petzl-Erler, M. L. (2015) KIR and HLA under pressure: evidences of coevolution across worldwide populations. *Hum Genet,* 134(9)**,** 929-40.

Bao, X., Wang, M., Zhou, H., Zhang, H., Wu, X. & He, J. (2015) Donor KIR genotype Bx1 had a negative effect while centromeric B-specific gene motifs had a positive effect of survival for standard risk AML Patients after unrelated donor hemotopoietic stem cell transplantation. *Human Immunology,* 76.

Barbarin, A., Cayssials, E., Jacomet, F., Nunez, N. G., Basbous, S., Lefevre, L., Abdallah, M., Piccirilli, N., Morin, B., Lavoue, V., Catros, V., Piaggio, E., Herbelin, A. & Gombert, J. M. (2017) Phenotype of NK-Like CD8(+) T Cells with Innate Features in Humans and Their Relevance in Cancer Diseases. *Front Immunol,* 8**,** 316.

Bari, R., Bell, T., Leung, W. H., Vong, Q. P., Chan, W. K., Das Gupta, N., Holladay, M., Rooney, B. & Leung, W. (2009) Significant functional heterogeneity among KIR2DL1 alleles and a pivotal role of arginine 245. *Blood,* 114(25)**,** 5182-90.

Bari, R., Leung, M., Turner, V. E., Embrey, C., Rooney, B., Holladay, M. & Leung, W. (2011) Molecular determinant-based typing of KIR alleles and KIR ligands. *Clin Immunol,* 138(3)**,** 274-81.

Bari, R., Rujkijyanont, P., Sullivan, E., Kang, G., Turner, V., Gan, K. & Leung, W. (2013) Effect of donor KIR2DL1 allelic polymorphism on the outcome of pediatric allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol,* 31(30)**,** 3782-90.

Baron, F. & Sandmaier, B. M. (2006) Chimerism and outcomes after allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning. *Leukemia,* 20(10)**,** 1690-700.

Bell, J. C. & Kowalczykowski, S. C. (2016) RecA: Regulation and Mechanism of a Molecular Search Engine. *Trends Biochem Sci,* 41(6)**,** 491-507.

Béziat, V., Traherne, J. A., Liu, L. L., Jayaraman, J., Enqvist, M., Larsson, S., Trowsdale, J. & Malmberg, K. J. (2013) Influence of KIR gene copy number on natural killer cell education. *Blood,* 121(23)**,** 4703-7.

Bjorkstrom, N. K., Beziat, V., Cichocki, F., Liu, L. L., Levine, J., Larsson, S., Koup, R. A., Anderson, S. K., Ljunggren, H. G. & Malmberg, K. J. (2012) CD8 T cells express randomly selected KIRs with distinct specificities compared with NK cells. *Blood,* 120(17)**,** 3455-65.

Boelen, L., Debebe, B., Silveira, M., Salam, A., Makinde, J., Roberts, C. H., Wang, E. C. Y., Frater, J., Gilmour, J., Twigger, K., Ladell, K., Miners, K. L., Jayaraman, J., Traherne, J. A., Price, D. A., Qi, Y., Martin, M. P., Macallan, D. C., Thio, C. L., Astemborski, J., Kirk, G., Donfield, S. M., Buchbinder, S., Khakoo, S. I., Goedert, J. J., Trowsdale, J., Carrington, M., Kollnberger, S. & Asquith, B. (2018) Inhibitory killer cell immunoglobulin-like receptors strengthen CD8(+) T cell-mediated control of HIV-1, HCV, and HTLV-1. *Sci Immunol,* 3(29)**,** eaao2892.

Boudreau, J. E., Giglio, F., Gooley, T. A., Stevenson, P. A., Le Luduec, J. B., Shaffer, B. C., Rajalingam, R., Hou, L., Hurley, C. K., Noreen, H., Reed, E. F., Yu, N., Vierra-Green, C., Haagenson, M., Malkki, M., Petersdorf, E. W., Spellman, S. & Hsu, K. C. (2017) KIR3DL1/HLA-B Subtypes Govern Acute Myelogenous Leukemia Relapse After Hematopoietic Cell Transplantation. *J Clin Oncol,* 35(20)**,** 2268-2278.

Boudreau, J. E. & Hsu, K. C. (2018) Natural Killer Cell Education and the Response to Infection and Cancer Therapy: Stay Tuned. *Trends Immunol,* 39(3)**,** 222-239.

Boudreau, J. E., Le Luduec, J. B. & Hsu, K. C. (2014) Development of a novel multiplex PCR assay to detect functional subtypes of KIR3DL1 alleles. *PLoS One,* 9(6)**,** e99543.

Boudreau, J. E., Mulrooney, T. J., Le Luduec, J. B., Barker, E. & Hsu, K. C. (2016) KIR3DL1 and HLA-B density and binding calibrate NK education and response to HIV. *J Immunol,* 196(8)**,** 3398-410.

Boyington, J. C., Motyka, S. A., Schuck, P., Brooks, A. G. & Sun, P. D. (2000) Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. *Nature,* 405(6786)**,** 537-43.

Brelsford, J. (2016) *NMDP-bioinformatics/DaSH Home.* [Online]. Available: https://github.com/nmdp-bioinformatics/dash/wiki [Accessed 16/05/2019].

Broder, M. S., Quock, T. P., Chang, E., Reddy, S. R., Agarwal-Hashmi, R., Arai, S. & Villa, K. F. (2017) The cost of hematopoietic stem-cell transplantation in the United States. *Am Health Drug Benefits,* 10(7)**,** 366-374.

Bultitude, W. P., Gymer, A. W., Robinson, J., Anthias, C., Potter, M. N., Russell, N., Pagliuca, A., Potter, V. T., Bloor, A. J., Marks, D., Patel, A., MacKinnon, S., Carpenter, B., McQuaker, I. G., Szydlo, R. M., Perry, J., Wilson, M., Mayor, N. P. & Marsh, S. G. E. (Year) The effect of donor KIR2DL1 allelic diversity on the outcomes of HSCT is influenced by conditioning regimen. *In:* KIR Workshop 25th-27th October 2018 21/07/2019 2018 Camogli (GE) Italy. HLA.

Bultitude, W. P., Schellekens, J., Szydlo, R. M., Anthias, C., Cooley, S. A., Miller, J. S., Weisdorf, D. J., Shaw, B. E., Roberts, C. H., Garcia-Sepulveda, C. A., Lee, J., Pearce, R. M., Wilson, M. C., Potter, M. N., Byrne, J. L., Russell, N. H., MacKinnon, S., Bloor, A. J., Patel, A., McQuaker, I. G., Malladi, R., Tholouli, E., Orchard, K., Potter, V. T., Madrigal, J. A., Mayor, N. P. & Marsh, S. G. E. (2020) Presence of donor-encoded centromeric KIR B content increases the risk of infectious mortality in recipients of myeloablative, T-cell deplete, HLA-matched HCT to treat AML. *Bone Marrow Transplant.*

Burek Kamenaric, M., Stingl Jankovic, K., Grubic, Z., Serventi Seiwerth, R., Maskalan, M., Nemet, D., Mikulic, M. & Zunec, R. (2017) The impact of KIR2DS4 gene on clinical outcome after hematopoietic stem cell transplantation. *Hum Immunol,* 78(2)**,** 95-102.

Burek, M. K., Grubic, Z., Stingl, K. & Zunec, R. (2013) Distribution of KIR genes in the Croatian population. *Hum Immunol,* 74(8)**,** 952-6.

Burian, A., Wang, K. L., Finton, K. A., Lee, N., Ishitani, A., Strong, R. K. & Geraghty, D. E. (2016) HLA-F and MHC-I open conformers bind natural killer cell Ig-like receptor KIR3DS1. *PLoS One,* 11(9)**,** e0163297.

Campbell, K. S. & Hasegawa, J. (2013) Natural killer cell biology: an update and future directions. *J Allergy Clin Immunol,* 132(3)**,** 536-544.

Canter, R. J., Grossenbacher, S. K., Foltz, J. A., Sturgill, I. R., Park, J. S., Luna, J. I., Kent, M. S., Culp, W. T. N., Chen, M., Modiano, J. F., Monjazeb, A. M., Lee, D. A. & Murphy, W. J. (2017) Radiotherapy enhances natural killer cell cytotoxicity and localization in pre-clinical canine sarcomas and first-in-dog clinical trial. *J Immunother Cancer,* 5(1)**,** 98.

Carapito, R., Radosavljevic, M. & Bahram, S. (2016) Next-generation sequencing of the HLA locus: methods and impacts on HLA typing, population genetics and disease association studies. *Hum Immunol,* 77(11)**,** 1016-23.

Cella, M., Longo, A., Ferrara, G. B., Strominger, J. L. & Colonna, M. (1994) NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. *J Exp Med,* 180(4)**,** 1235-42.

Chan, H. W., Kurago, Z. B., Stewart, C. A., Wilson, M. J., Martin, M. P., Mace, B. E., Carrington, M., Trowsdale, J. & Lutz, C. T. (2003) DNA methylation

maintains allele-specific KIR gene expression in human natural killer cells. *J Exp Med,* 197(2), 245-55.

Chan, Y. L. T., Zuo, J., Inman, C., Croft, W., Begum, J., Croudace, J., Kinsella, F., Maggs, L., Nagra, S., Nunnick, J., Abbotts, B., Craddock, C., Malladi, R. & Moss, P. (2018) NK cells produce high levels of IL-10 early after allogeneic stem cell transplantation and suppress development of acute GVHD. *Eur J Immunol,* 48(2), 316-329.

Chen, Y., Shi, Y., Cheng, H., An, Y. Q. & Gao, G. F. (2009) Structural immunology and crystallography help immunologists see the immune system in action: how T and NK cells touch their ligands. *IUBMB Life,* 61(6), 579-90.

Ciccone, E., Pende, D., Nanni, L., Di Donato, C., Viale, O., Beretta, A., Vitale, M., Sivori, S., Moretta, A. & Moretta, L. (1995) General role of HLA class I molecules in the protection of target cells from lysis by natural killer cells: evidence that the free heavy chains of class I molecules are not sufficient to mediate the protective effect. *Int Immunol,* 7(3), 393-400.

Cichocki, F., Miller, J. S. & Anderson, S. K. (2011) Killer immunoglobulin-like receptor transcriptional regulation: a fascinating dance of multiple promoters. *J Innate Immun,* 3(3), 242-8.

Cisneros, E., Moraru, M., Gómez-Lozano, N., Muntasell, A., López-Botet, M. & Vilches, C. (2020) Haplotype-Based Analysis of KIR-Gene Profiles in a South European Population—Distribution of Standard and Variant Haplotypes, and Identification of Novel Recombinant Structures. *Frontiers in Immunology,* 11.

Closa, L., Vidal, F., Herrero, M. J. & Caro, J. L. (2020) Distribution of human killer cell immunoglobulin-like receptors and ligands among blood donors of Catalonia. *HLA,* 95(3), 179-188.

Colonna, M., Borsellino, G., Falco, M., Ferrara, G. B. & Strominger, J. L. (1993) HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci U S A,* 90(24), 12000-4.

Colonna, M., Spies, T., Strominger, J. L., Ciccone, E., Moretta, A., Moretta, L., Pende, D. & Viale, O. (1992) Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene. *Proc Natl Acad Sci U S A,* 89(17), 7983-5.

Cooley, S., He, F., Bachanova, V., Vercellotti, G. M., DeFor, T. E., Curtsinger, J. M., Robertson, P., Grzywacz, B., Conlon, K. C., Waldmann, T. A., McKenna, D. H., Blazar, B. R., Weisdorf, D. J. & Miller, J. S. (2019) First-in-human trial of rhIL-15 and haploidentical natural killer cell therapy for advanced acute myeloid leukemia. *Blood Adv,* 3(13), 1970-1980.

Cooley, S., Trachtenberg, E., Bergemann, T. L., Saeteurn, K., Klein, J., Le, C. T., Marsh, S. G., Guethlein, L. A., Parham, P., Miller, J. S. & Weisdorf, D. J. (2009) Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood,* 113(3), 726-32.

Cooley, S., Weisdorf, D. J., Guethlein, L. A., Klein, J. P., Wang, T., Le, C. T., Marsh, S. G., Geraghty, D., Spellman, S., Haagenson, M. D., Ladner, M., Trachtenberg, E., Parham, P. & Miller, J. S. (2010) Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood,* 116(14), 2411-9.

Cooley, S., Weisdorf, D. J., Guethlein, L. A., Klein, J. P., Wang, T., Marsh, S. G., Spellman, S., Haagenson, M. D., Saeturn, K., Ladner, M., Trachtenberg, E.,

Parham, P. & Miller, J. S. (2014) Donor killer cell Ig-like receptor B haplotypes, recipient HLA-C1, and HLA-C mismatch enhance the clinical benefit of unrelated transplantation for acute myelogenous leukemia. *J Immunol,* 192(10), 4592-600.

Crocchiolo, R., Zino, E., Vago, L., Oneto, R., Bruno, B., Pollichieni, S., Sacchi, N., Sormani, M. P., Marcon, J., Lamparelli, T., Fanin, R., Garbarino, L., Miotti, V., Bandini, G., Bosi, A., Ciceri, F., Bacigalupo, A., Fleischhauer, K., Gruppo Italiano Trapianto di Midollo Osseo, C. S. E. e. T. C. & Italian Bone Marrow Donor, R. (2009) Nonpermissive HLA-DPB1 disparity is a significant independent risk factor for mortality after unrelated hematopoietic stem cell transplantation. *Blood,* 114(7), 1437-44.

Davies, G. E., Locke, S. M., Wright, P. W., Li, H., Hanson, R. J., Miller, J. S. & Anderson, S. K. (2007) Identification of bidirectional promoters in the human KIR genes. *Genes Immun,* 8(3), 245-53.

Davies, S. M., Iannone, R., Alonzo, T. A., Wang, Y. C., Gerbing, R., Soni, S., Kolb, E. A., Meshinchi, S., Orchard, P. J., Burns, L. J., Shenoy, S. & Leung, W. (2020) A Phase 2 Trial of KIR-Mismatched Unrelated Donor Transplantation Using in Vivo T Cell Depletion with Antithymocyte Globulin in Acute Myelogenous Leukemia: Children's Oncology Group AAML05P1 Study. *Biol Blood Marrow Transplant,* 26(4), 712-717.

Dehn, J., Spellman, S., Hurley, C. K., Shaw, B. E., Barker, J. N., Burns, L. J., Confer, D. L., Eapen, M., Fernandez-Vina, M., Hartzman, R., Maiers, M., Marino, S. R., Mueller, C., Perales, M. A., Rajalingam, R. & Pidala, J. (2019) Selection of unrelated donors and cord blood units for hematopoietic cell transplantation: guidelines from the NMDP/CIBMTR. *Blood,* 134(12), 924-934.

Dulberger, C. L., McMurtrey, C. P., Holzemer, A., Neu, K. E., Liu, V., Steinbach, A. M., Garcia-Beltran, W. F., Sulak, M., Jabri, B., Lynch, V. J., Altfeld, M., Hildebrand, W. H. & Adams, E. J. (2017) Human leukocyte antigen F presents peptides and regulates immunity through interactions with NK cell receptors. *Immunity,* 46(6), 1018-29 e7.

Elliott, J. M. & Yokoyama, W. M. (2011) Unifying concepts of MHC-dependent natural killer cell education. *Trends Immunol,* 32(8), 364-72.

EMBL-EBI. (2017) *Donor KIR B-content group calculator* [Online]. Available: https://www.ebi.ac.uk/ipd/kir/donor_b_content.html [Accessed 16/05/2019].

EMBL-EBI. (2019) *Statistics for release 2.9.0 (December 2019)* [Online]. Available: https://www.ebi.ac.uk/ipd/kir/stats.html [Accessed 16/05/2019].

Falco, M., Sivori, S., Meazza, R. & Pende, D. (2019) Report from the Eleventh Killer Immunoglobulin-like Receptor (KIR) Workshop: Novel insights on KIR polymorphism, ligand recognition, expression and function. *HLA,* 94(2), 100-110.

Fan, Q. R., Garboczi, D. N., Winter, C. C., Wagtmann, N., Long, E. O. & Wiley, D. C. (1996) Direct binding of a soluble natural killer cell inhibitory receptor to a soluble human leukocyte antigen-Cw4 class I major histocompatibility complex molecule. *Proc Natl Acad Sci U S A,* 93(14), 7178-83.

Fan, Q. R., Long, E. O. & Wiley, D. C. (2001) Crystal structure of the human natural killer cell inhibitory receptor KIR2DL1-HLA-Cw4 complex. *Nat Immunol,* 2(5), 452-60.

Fan, Q. R., Mosyak, L., Winter, C. C., Wagtmann, N., Long, E. O. & Wiley, D. C. (1997) Structure of the inhibitory receptor for human natural killer cells resembles haematopoietic receptors. *Nature,* 389(6646), 96-100.

Fan, Q. R. & Wiley, D. C. (1999) Structure of human histocompatibility leukocyte antigen (HLA)-Cw4, a ligand for the KIR2D natural killer cell inhibitory receptor. *J Exp Med,* 190(1), 113-23.

Faridi, R. M., Kemp, T. J., Dharmani-Khan, P., Lewis, V., Tripathi, G., Rajalingam, R., Daly, A., Berka, N., Storek, J. & Masood Khan, F. (2016) Donor-Recipient Matching for KIR Genotypes Reduces Chronic GVHD and Missing Inhibitory KIR Ligands Protect against Relapse after Myeloablative, HLA Matched Hematopoietic Cell Transplantation. *PLoS One,* 11(6), e0158242.

Fischer, J. C., Ottinger, H., Ferencik, S., Sribar, M., Punzel, M., Beelen, D. W., Schwan, M. A., Grosse-Wilde, H., Wernet, P. & Uhrberg, M. (2007) Relevance of C1 and C2 epitopes for hemopoietic stem cell transplantation: role for sequential acquisition of HLA-C-specific inhibitory killer Ig-like receptor. *The Journal of Immunology,* 178(6), 3918-23.

Fleischhauer, K., Fernandez-Vina, M. A., Wang, T., Haagenson, M., Battiwalla, M., Baxter-Lowe, L. A., Ciceri, F., Dehn, J., Gajewski, J., Hale, G. A., Heemskerk, M. B., Marino, S. R., McCarthy, P. L., Miklos, D., Oudshoorn, M., Pollack, M. S., Reddy, V., Senitzer, D., Shaw, B. E., Waller, E. K., Lee, S. J. & Spellman, S. R. (2014) Risk associations between HLA-DPB1 T-cell epitope matching and outcome of unrelated hematopoietic cell transplantation are independent of HLA-DPA1. *Bone Marrow Transplant,* 49(9), 1176-83.

Fleischhauer, K., Kernan, N. A., O'Reilly, R. J., Dupont, B. & Yang, S. Y. (1990) Bone Marrow–Allograft Rejection by T Lymphocytes Recognizing a Single Amino Acid Difference in HLA-B44. *New England Journal of Medicine,* 323(26), 1818-1822.

Forlenza, C. J., Boudreau, J. E., Zheng, J., Le Luduec, J. B., Chamberlain, E., Heller, G., Cheung, N. K. & Hsu, K. C. (2016) KIR3DL1 Allelic Polymorphism and HLA-B Epitopes Modulate Response to Anti-GD2 Monoclonal Antibody in Patients With Neuroblastoma. *J Clin Oncol,* 34(21), 2443-51.

Frazier, W. R., Steiner, N., Hou, L., Dakshanamurthy, S. & Hurley, C. K. (2013) Allelic variation in KIR2DL3 generates a KIR2DL2-like receptor with increased binding to its HLA-C ligand. *J Immunol,* 190(12), 6198-208.

Gaetano, J. (2018) *Holm-Bonferroni sequential correction: An Excel calculator (1.3) [Microsoft Excel Workbook]* [Online]. Available: https://www.researchgate.net/publication/322568540_Holm-Bonferroni_sequential_correction_An_Excel_calculator_13 [Accessed].

Gamazon, E. R. & Stranger, B. E. (2015) The impact of human copy number variation on gene expression. *Brief Funct Genomics,* 14(5), 352-7.

Gendzekhadze, K., Garcia-Gomez, J., Cano, P. & Senitzer, D. (2015) KIR shapes HLA. *Human Immunology,* 76, 40.

Gentle, N. L., Loubser, S., Paximadis, M., Puren, A. & Tiemessen, C. T. (2017) Killer-cell immunoglobulin-like receptor (KIR) and human leukocyte antigen (HLA) class I genetic diversity in four South African populations. *Hum Immunol,* 78(7-8), 503-509.

Gianchecchi, E., Delfino, D. V. & Fierabracci, A. (2018) NK cells in autoimmune diseases: Linking innate and adaptive immune responses. *Autoimmun Rev,* 17(2), 142-54.

Gomez-Lozano, N., Estefania, E., Williams, F., Halfpenny, I., Middleton, D., Solis, R. & Vilches, C. (2005) The silent KIR3DP1 gene (CD158c) is transcribed and might encode a secreted receptor in a minority of humans, in whom the

KIR3DP1, KIR2DL4 and KIR3DL1/KIR3DS1 genes are duplicated. *Eur J Immunol,* 35(1), 16-24.

Gonzalez-Galarza, F. F., Takeshita, L. Y., Santos, E. J., Kempson, F., Maia, M. H., da Silva, A. L., Teles e Silva, A. L., Ghattaoraya, G. S., Alfirevic, A., Jones, A. R. & Middleton, D. (2015) Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. *Nucleic Acids Res,* 43(Database issue), D784-8.

Goodson-Gregg, F. J., Krepel, S. A. & Anderson, S. K. (2020) Tuning of human NK cells by endogenous HLA-C expression. *Immunogenetics,* 72(4), 205-215.

Gourraud, P. A., Meenagh, A., Cambon-Thomsen, A. & Middleton, D. (2010) Linkage disequilibrium organization of the human KIR superlocus: implications for KIR data analyses. *Immunogenetics,* 62(11-12), 729-40.

Guinan, K. J., Cunningham, R. T., Meenagh, A., Dring, M. M., Middleton, D. & Gardiner, C. M. (2010) Receptor systems controlling natural killer cell function are genetically stratified in Europe. *Genes Immun,* 11(1), 67-78.

Gumperz, J. E., Barber, L. D., Valiante, N. M., Percival, L., Phillips, J. H., Lanier, L. L. & Parham, P. (1997) Conserved and variable residues within the Bw4 motif of HLA-B make separable contributions to recognition by the NKB1 killer cell-inhibitory receptor. *J Immunol,* 158(11), 5237-41.

Gumperz, J. E., Litwin, V., Phillips, J. H., Lanier, L. L. & Parham, P. (1995) The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med,* 181(3), 1133-44.

Hansasuta, P., Dong, T., Thananchai, H., Weekes, M., Willberg, C., Aldemir, H., Rowland-Jones, S. & Braud, V. M. (2004) Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. *Eur J Immunol,* 34(6), 1673-9.

Hilton, H. G., Guethlein, L. A., Goyos, A., Nemat-Gorgani, N., Bushnell, D. A., Norman, P. J. & Parham, P. (2015a) Polymorphic HLA-C receptors balance the functional characteristics of KIR haplotypes. *J Immunol,* 195(7), 3160-70.

Hilton, H. G., Norman, P. J., Nemat-Gorgani, N., Goyos, A., Hollenbach, J. A., Henn, B. M., Gignoux, C. R., Guethlein, L. A. & Parham, P. (2015b) Loss and gain of natural killer cell receptor function in an African hunter-gatherer population. *PLoS Genet,* 11(8), e1005439.

Hölzemer, A., Thobakgale, C. F., Jimenez Cruz, C. A., Garcia-Beltran, W. F., Carlson, J. M., van Teijlingen, N. H., Mann, J. K., Jaggernath, M., Kang, S. G., Korner, C., Chung, A. W., Schafer, J. L., Evans, D. T., Alter, G., Walker, B. D., Goulder, P. J., Carrington, M., Hartmann, P., Pertel, T., Zhou, R., Ndung'u, T. & Altfeld, M. (2015) Selection of an HLA-C*03:04-Restricted HIV-1 p24 Gag Sequence Variant Is Associated with Viral Escape from KIR2DL3+ Natural Killer Cells: Data from an Observational Cohort in South Africa. *PLoS Med,* 12(11), e1001900; discussion e1001900.

Horowitz, A., Strauss-Albee, D. M., Leipold, M., Kubo, J., Nemat-Gorgani, N., Dogan, O. C., Dekker, C. L., Mackey, S., Maecker, H., Swan, G. E., Davis, M. M., Norman, P. J., Guethlein, L. A., Desai, M., Parham, P. & Blish, C. A. (2013) Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. *Sci Transl Med,* 5(208), 208ra145.

Hou, L., Chen, M., Steiner, N., Kariyawasam, K., Ng, J. & Hurley, C. K. (2012) Killer cell immunoglobulin-like receptors (KIR) typing by DNA sequencing. *Methods Mol Biol,* 882, 431-68.

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(3), 360-70.

Howard, C. A., Fernandez-Vina, M. A., Appelbaum, F. R., Confer, D. L., Devine, S. M., Horowitz, M. M., Mendizabal, A., Laport, G. G., Pasquini, M. C. & Spellman, S. R. (2015) Recommendations for donor human leukocyte antigen assessment and matching for allogeneic stem cell transplantation: consensus opinion of the Blood and Marrow Transplant Clinical Trials Network (BMT CTN). *Biol Blood Marrow Transplant,* 21(1), 4-7.

Hsu, K. C., Chida, S., Geraghty, D. E. & Dupont, B. (2002a) The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev,* 190, 40-52.

Hsu, K. C., Liu, X. R., Selvakumar, A., Mickelson, E., O'Reilly, R. J. & Dupont, B. (2002b) Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *The Journal of Immunology,* 169(9), 5118-29.

Huard, B. & Karlsson, L. (2000) KIR expression on self-reactive CD8+ T cells is controlled by T-cell receptor engagement. *Nature,* 403(6767), 325-8.

Ivarsson, M. A., Michaelsson, J. & Fauriat, C. (2014) Activating killer cell Ig-like receptors in health and disease. *Front Immunol,* 5, 184.

Jia, H., Guo, Y., Zhao, W. & Wang, K. (2014) Long-range PCR in next-generation sequencing: comparison of six enzymes and evaluation on the MiSeq sequencer. *Sci Rep,* 4(5737), 1-8.

Jiang, W., Johnson, C., Jayaraman, J., Simecek, N., Noble, J., Moffatt, M. F., Cookson, W. O., Trowsdale, J. & Traherne, J. A. (2012) Copy number variation leads to considerable diversity for B but not A haplotypes of the human KIR genes encoding NK cell receptors. *Genome Res,* 22(10), 1845-54.

Kärre, K., Ljunggren, H. G., Piontek, G. & Kiessling, R. (1986) Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature,* 319(6055), 675-8.

Khera, N., Zeliadt, S. B. & Lee, S. J. (2012) Economics of hematopoietic cell transplantation. *Blood,* 120(8), 1545-51.

Koopman, L. A., Kopcow, H. D., Rybalov, B., Boyson, J. E., Orange, J. S., Schatz, F., Masch, R., Lockwood, C. J., Schachter, A. D., Park, P. J. & Strominger, J. L. (2003) Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med,* 198(8), 1201-12.

Krasnova, Y., Putz, E. M., Smyth, M. J. & Souza-Fonseca-Guimaraes, F. (2017) Bench to bedside: NK cells and control of metastasis. *Clin Immunol,* 177, 50-59.

Krieger, E., Sabo, R., Moezzi, S., Cain, C., Roberts, C., Kimball, P., Chesney, A., McCarty, J., Keating, A., Romee, R., Wiedl, C., Qayyum, R. & Toor, A. (2020) Killer Immunoglobulin-Like Receptor-Ligand Interactions Predict Clinical Outcomes following Unrelated Donor Transplantations. *Biol Blood Marrow Transplant,* 26(4), 672-682.

Le Luduec, J. B., Boudreau, J. E., Freiberg, J. C. & Hsu, K. C. (2019) Novel Approach to Cell Surface Discrimination Between KIR2DL1 Subtypes and KIR2DS1

Identifies Hierarchies in NK Repertoire, Education, and Tolerance. *Front Immunol,* 10**,** 734.

Le Luduec, J. B., Kudva, A., Boudreau, J. E. & Hsu, K. C. (2018) Novel multiplex PCR-SSP method for centromeric KIR allele discrimination. *Sci Rep,* 8(1)**,** 14853.

Leung, W., Handgretinger, R., Iyengar, R., Turner, V., Holladay, M. S. & Hale, G. A. (2007) Inhibitory KIR-HLA receptor-ligand mismatch in autologous haematopoietic stem cell transplantation for solid tumour and lymphoma. *Br J Cancer,* 97(4)**,** 539-42.

Leung, W., Iyengar, R., Turner, V., Lang, P., Bader, P., Conn, P., Niethammer, D. & Handgretinger, R. (2004) Determinants of antileukemia effects of allogeneic NK cells. *The Journal of Immunology,* 172(1)**,** 644-50.

Li, W. & Freudenberg, J. (2014) Mappability and read length. *Front Genet,* 5(381)**,** 1-7.

Li, W., Freudenberg, J. & Miramontes, P. (2014) Diminishing return for increased mappability with longer sequencing reads: implications of the k-mer distributions in the human genome. *BMC Bioinformatics,* 15(2)**,** 1-12.

Li, Y. & Mariuzza, R. A. (2014) Structural basis for recognition of cellular and viral ligands by NK cell receptors. *Front Immunol,* 5**,** 123.

Lisnic, B., Lisnic, V. J. & Jonjic, S. (2015) NK cell interplay with cytomegaloviruses. *Curr Opin Virol,* 15**,** 9-18.

Little, A. M., Green, A., Harvey, J., Hemmatpour, S., Latham, K., Marsh, S. G., Poulton, K. & Sage, D. (2016) BSHI Guidelines: HLA matching and donor selection for haematopoietic progenitor cell transplantation. *Int J Immunogenet,* 43(5)**,** 263-86.

Liu, J., Xiao, Z., Ko, H. L., Shen, M. & Ren, E. C. (2014) Activating killer cell immunoglobulin-like receptor 2DS2 binds to HLA-A*11. *Proc Natl Acad Sci U S A,* 111(7)**,** 2662-7.

Lunemann, S., Martrus, G., Holzemer, A., Chapel, A., Ziegler, M., Korner, C., Garcia Beltran, W., Carrington, M., Wedemeyer, H. & Altfeld, M. (2016) Sequence variations in HCV core-derived epitopes alter binding of KIR2DL3 to HLA-C *03:04 and modulate NK cell function. *J Hepatol,* 65(2)**,** 252-8.

Maccari, G., Robinson, J., Hammond, J. A. & Marsh, S. G. E. (2020) The IPD Project: a centralised resource for the study of polymorphism in genes of the immune system. *Immunogenetics,* 72(1-2)**,** 49-55.

Machado-Sulbaran, A. C., Munoz-Valle, J. F., Ramirez-Duenas, M. G., Banos-Hernandez, C. J., Graciano-Machuca, O., Velarde-De la Cruz, E. E., Parra-Rojas, I. & Sanchez-Hernandez, P. E. (2017) Distribution of KIR genes and KIR2DS4 gene variants in two Mexican Mestizo populations. *Hum Immunol,* 78(10)**,** 614-620.

Mack, S. J., Cano, P., Hollenbach, J. A., He, J., Hurley, C. K., Middleton, D., Moraes, M. E., Pereira, S. E., Kempenich, J. H., Reed, E. F., Setterholm, M., Smith, A. G., Tilanus, M. G., Torres, M., Varney, M. D., Voorter, C. E., Fischer, G. F., Fleischhauer, K., Goodridge, D., Klitz, W., Little, A. M., Maiers, M., Marsh, S. G., Muller, C. R., Noreen, H., Rozemuller, E. H., Sanchez-Mazas, A., Senitzer, D., Trachtenberg, E. & Fernandez-Vina, M. (2013) Common and well-documented HLA alleles: 2012 update to the CWD catalogue. *Tissue Antigens,* 81(4)**,** 194-203.

Mack, S. J., Milius, R. P., Gifford, B. D., Sauter, J., Hofmann, J., Osoegawa, K., Robinson, J., Groeneweg, M., Turenchalk, G. S., Adai, A., Holcomb, C.,

Rozemuller, E. H., Penning, M. T., Heuer, M. L., Wang, C., Salit, M. L., Schmidt, A. H., Parham, P. R., Muller, C., Hague, T., Fischer, G., Fernandez-Vina, M., Hollenbach, J. A., Norman, P. J. & Maiers, M. (2015) Minimum information for reporting next generation sequence genotyping (MIRING): guidelines for reporting HLA and KIR genotyping via next generation sequencing. *Hum Immunol,* 76(12), 954-62.

Maiers, M., Gragert, L. & Klitz, W. (2007) High-resolution HLA alleles and haplotypes in the United States population. *Hum Immunol,* 68(9), 779-88.

Mandelboim, O., Reyburn, H. T., Valés-Gómez, M., Pazmany, L., Colonna, M., Borsellino, G. & Strominger, J. L. (1996) Protection from lysis by Natural Killer cells of group 1 and 2 specificity is mediated by residue 80 in human Histocompatibility Leukocyte Antigen C alleles and also occurs with empty Major Histocompatibility Complex molecules. *J Exp Med,* 184, 913-22.

Manser, A. R., Weinhold, S. & Uhrberg, M. (2015) Human KIR repertoires: shaped by genetic diversity and evolution. *Immunol Rev,* 267(1), 178-96.

Marsh, S. G., Parham, P., Dupont, B., Geraghty, D. E., Trowsdale, J., Middleton, D., Vilches, C., Carrington, M., Witt, C., Guethlein, L. A., Shilling, H., Garcia, C. A., Hsu, K. C. & Wain, H. (2003) Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Immunogenetics,* 55(4), 220-6.

Martin, M. P., Bashirova, A., Traherne, J., Trowsdale, J. & Carrington, M. (2003) Cutting edge: expansion of the KIR locus by unequal crossing over. *The Journal of Immunology,* 171(5), 2192-5.

Martin, M. P., Gao, X., Lee, J. H., Nelson, G. W., Detels, R., Goedert, J. J., Buchbinder, S., Hoots, K., Vlahov, D., Trowsdale, J., Wilson, M., O'Brien, S. J. & Carrington, M. (2002) Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet,* 31(4), 429-34.

Martin, M. P., Naranbhai, V., Shea, P. R., Qi, Y., Ramsuran, V., Vince, N., Gao, X., Thomas, R., Brumme, Z. L., Carlson, J. M., Wolinsky, S. M., Goedert, J. J., Walker, B. D., Segal, F. P., Deeks, S. G., Haas, D. W., Migueles, S. A., Connors, M., Michael, N., Fellay, J., Gostick, E., Llewellyn-Lacey, S., Price, D. A., Lafont, B. A., Pymm, P., Saunders, P. M., Widjaja, J., Wong, S. C., Vivian, J. P., Rossjohn, J., Brooks, A. G. & Carrington, M. (2018) Killer cell immunoglobulin-like receptor 3DL1 variation modifies HLA-B*57 protection against HIV-1. *J Clin Invest,* 128(5), 1903-1912.

Martin, M. P., Qi, Y., Gao, X., Yamada, E., Martin, J. N., Pereyra, F., Colombo, S., Brown, E. E., Shupert, W. L., Phair, J., Goedert, J. J., Buchbinder, S., Kirk, G. D., Telenti, A., Connors, M., O'Brien, S. J., Walker, B. D., Parham, P., Deeks, S. G., McVicar, D. W. & Carrington, M. (2007) Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet,* 39(6), 733-40.

Maxwell, L. D., Wallace, A., Middleton, D. & Curran, M. D. (2002) A common KIR2DS4 deletion variant in the human that predicts a soluble KIR molecule analogous to the KIR1D molecule observed in the rhesus monkey. *Tissue Antigens,* 60(3), 254-8.

Mayor, N. P., Hayhurst, J. D., Turner, T. R., Szydlo, R. M., Shaw, B. E., Bultitude, W. P., Sayno, J. R., Tavarozzi, F., Latham, K., Anthias, C., Robinson, J., Braund, H., Danby, R., Perry, J., Wilson, M. C., Bloor, A. J., McQuaker, I. G., MacKinnon, S., Marks, D. I., Pagliuca, A., Potter, M. N., Potter, V. T., Russell, N. H., Thomson, K. J., Madrigal, J. A. & Marsh, S. G. E. (2019) Recipients Receiving Better HLA-Matched Hematopoietic Cell Transplantation Grafts,

Uncovered by a Novel HLA Typing Method, Have Superior Survival: A Retrospective Study. *Biol Blood Marrow Transplant,* 25(3), 443-450.

Middleton, D., Gonzalez, A. & Gilmore, P. M. (2007) Studies on the expression of the deleted KIR2DS4*003 gene product and distribution of KIR2DS4 deleted and nondeleted versions in different populations. *Hum Immunol,* 68(2), 128-34.

Milius, R. P., Heuer, M., Valiga, D., Doroschak, K. J., Kennedy, C. J., Bolon, Y. T., Schneider, J., Pollack, J., Kim, H. R., Cereb, N., Hollenbach, J. A., Mack, S. J. & Maiers, M. (2015) Histoimmunogenetics Markup Language 1.0: reporting next generation sequencing-based HLA and KIR genotyping. *Hum Immunol,* 76(12), 963-74.

Miller, J. S. & McCullar, V. (2001) Human natural killer cells with polyclonal lectin and immunoglobulinlike receptors develop from single hematopoietic stem cells with preferential expression of NKG2A and KIR2DL2/L3/S2. *Blood,* 98(3), 705-13.

Minculescu, L., Marquart, H. V., Friis, L. S., Petersen, S. L., Schiodt, I., Ryder, L. P., Andersen, N. S. & Sengeloev, H. (2016) Early Natural Killer Cell Reconstitution Predicts Overall Survival in T Cell-Replete Allogeneic Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant,* 22(12), 2187-2193.

Misra, M. K., Augusto, D. G., Martin, G. M., Nemat-Gorgani, N., Sauter, J., Hofmann, J. A., Traherne, J. A., Gonzalez-Quezada, B., Gorodezky, C., Bultitude, W. P., Marin, W., Vierra-Green, C., Anderson, K. M., Balas, A., Caro-Oleas, J. L., Cisneros, E., Colucci, F., Dandekar, R., Elfishawi, S. M., Fernandez-Vina, M. A., Fouda, M., Gonzalez-Fernandez, R., Grosse, A., Herrero-Mata, M. J., Hollenbach, S. Q., Marsh, S. G. E., Mentzer, A., Middleton, D., Moffett, A., Moreno-Hidalgo, M. A., Mossallam, G. I., Nakimuli, A., Oksenberg, J. R., Oppenheimer, S. J., Parham, P., Petzl-Erler, M. L., Planelles, D., Sanchez-Garcia, F., Sanchez-Gordo, F., Schmidt, A. H., Trowsdale, J., Vargas, L. B., Vicario, J. L., Vilches, C., Norman, P. J. & Hollenbach, J. A. (2018) Report from the killer-cell immunoglobulin-like receptors (KIR) component of the 17th International HLA and Immunogenetics Workshop. *Hum Immunol,* 79(12), 825-33.

Moesta, A. K., Norman, P. J., Yawata, M., Yawata, N., Gleimer, M. & Parham, P. (2008) Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *The Journal of Immunology,* 180(6), 3969-79.

Moretta, A., Sivori, S., Vitale, M., Pende, D., Morelli, L., Augugliaro, R., Bottino, C. & Moretta, L. (1995) Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. *J Exp Med,* 182(3), 875-84.

Mulrooney, T. J., Hou, L., Steiner, N. K., Chen, M., Belle, I., Ng, J. & Hurley, C. K. (2008) Promoter variants of KIR2DL5 add to diversity and may impact gene expression. *Immunogenetics,* 60(6), 287-94.

Murphy, W. J., Bennett, M., Kumar, V. & Longo, D. L. (1992) Donor-type activated natural killer cells promote marrow engraftment and B cell development during allogeneic bone marrow transplantation. *J Immunol,* 148(9), 2953-60.

Murphy, W. J., Kumar, V. & Bennett, M. (1987) Rejection of bone marrow allografts by mice with severe combined immune deficiency (SCID). Evidence that natural killer cells can mediate the specificity of marrow graft rejection. *J Exp Med,* 165(4), 1212-7.

Murphy, W. J., Kumar, V. & Bennett, M. (1990) Natural killer cells activated with interleukin 2 in vitro can be adoptively transferred and mediate hematopoietic histocompatibility-1 antigen-specific bone marrow rejection in vivo. *Eur J Immunol,* 20(8), 1729-34.

Muyas, F., Bosio, M., Puig, A., Susak, H., Domenech, L., Escaramis, G., Zapata, L., Demidov, G., Estivill, X., Rabionet, R. & Ossowski, S. (2019) Allele balance bias identifies systematic genotyping errors and false disease associations. *Hum Mutat,* 40(1), 115-126.

Nash, W. T., Teoh, J., Wei, H., Gamache, A. & Brown, M. G. (2014) Know Thyself: NK-Cell Inhibitory Receptors Prompt Self-Tolerance, Education, and Viral Control. *Front Immunol,* 5, 175.

Nelson, W. C., Pyo, C. W., Vogan, D., Wang, R., Pyon, Y. S., Hennessey, C., Smith, A., Pereira, S., Ishitani, A. & Geraghty, D. E. (2015) An integrated genotyping approach for HLA and other complex genetic systems. *Hum Immunol,* 76(12), 928-38.

Neuchel, C., Furst, D., Niederwieser, D., Bunjes, D., Tsamadou, C., Wulf, G., Pfreundschuh, M., Wagner, E., Stuhler, G., Einsele, H., Schrezenmeier, H. & Mytilineos, J. (2017) Impact of donor activating KIR genes on HSCT outcome in C1-ligand negative myeloid disease patients transplanted with unrelated donors - a retrospective study. *PLoS One,* 12(1), e0169512.

Neujahr, E., Schmidt, A. H. & Geist, S. (2020) DKMS Annual Report 2018.

Nguyen, S., Dhedin, N., Vernant, J. P., Kuentz, M., Al Jijakli, A., Rouas-Freiss, N., Carosella, E. D., Boudifa, A., Debre, P. & Vieillard, V. (2005) NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. *Blood,* 105(10), 4135-42.

Norman, P. J., Abi-Rached, L., Gendzekhadze, K., Hammond, J. A., Moesta, A. K., Sharma, D., Graef, T., McQueen, K. L., Guethlein, L. A., Carrington, C. V., Chandanayingyong, D., Chang, Y. H., Crespi, C., Saruhan-Direskeneli, G., Hameed, K., Kamkamidze, G., Koram, K. A., Layrisse, Z., Matamoros, N., Mila, J., Park, M. H., Pitchappan, R. M., Ramdath, D. D., Shiau, M. Y., Stephens, H. A., Struik, S., Tyan, D., Verity, D. H., Vaughan, R. W., Davis, R. W., Fraser, P. A., Riley, E. M., Ronaghi, M. & Parham, P. (2009) Meiotic recombination generates rich diversity in NK cell receptor genes, alleles, and haplotypes. *Genome Res,* 19(5), 757-69.

Norman, P. J., Hollenbach, J. A., Nemat-Gorgani, N., Marin, W. M., Norberg, S. J., Ashouri, E., Jayaraman, J., Wroblewski, E. E., Trowsdale, J., Rajalingam, R., Oksenberg, J. R., Chiaroni, J., Guethlein, L. A., Traherne, J. A., Ronaghi, M. & Parham, P. (2016) Defining KIR and HLA class I genotypes at highest resolution via high-throughput sequencing. *Am J Hum Genet,* 99(2), 375-91.

Nowak, J., Koscinska, K., Mika-Witkowska, R., Rogatko-Koros, M., Mizia, S., Jaskula, E., Polak, M., Mordak-Domagala, M., Lange, J., Gronkowska, A., Jedrzejczak, W. W., Kyrcz-Krzemien, S., Markiewicz, M., Dzierzak-Mietla, M., Tomaszewska, A., Nasilowska-Adamska, B., Szczepinski, A., Halaburda, K., Hellmann, A., Czyz, A., Gil, L., Komarnicki, M., Wachowiak, J., Baranska, M., Kowalczyk, J., Drabko, K., Gozdzik, J., Wysoczanska, B., Bogunia-Kubik, K., Graczyk-Pol, E., Witkowska, A., Marosz-Rudnicka, A., Nestorowicz, K., Dziopa, J., Szlendak, U., Warzocha, K., Lange, A. & Polish Donor-Recipient Matching Study, G. (2015) Role of donor activating KIR-HLA ligand-mediated NK cell education status in control of malignancy in

hematopoietic cell transplant recipients. *Biol Blood Marrow Transplant,* 21(5), 829-39.

Nowak, J., Koscinska, K., Mika-Witkowska, R., Rogatko-Koros, M., Mizia, S., Jaskula, E., Polak, M., Mordak-Domagala, M., Lange, J., Gronkowska, A., Jedrzejczak, W. W., Kyrcz-Krzemien, S., Markiewicz, M., Dzierzak-Mietla, M., Tomaszewska, A., Nasilowska-Adamska, B., Szczepinski, A., Halaburda, K., Hellmann, A., Komarnicki, M., Gil, L., Czyz, A., Wachowiak, J., Baranska, M., Kowalczyk, J., Drabko, K., Gozdzik, J., Wysoczanska, B., Bogunia-Kubik, K., Graczyk-Pol, E., Witkowska, A., Marosz-Rudnicka, A., Nestorowicz, K., Dziopa, J., Szlendak, U., Warzocha, K., Lange, A. A. & Polish Donor-Recipient Matching Study, G. (2014) Donor NK cell licensing in control of malignancy in hematopoietic stem cell transplant recipients. *Am J Hematol,* 89(10), E176-83.

O'Connor, G. M., Vivian, J. P., Gostick, E., Pymm, P., Lafont, B. A., Price, D. A., Rossjohn, J., Brooks, A. G. & McVicar, D. W. (2015) Peptide-dependent recognition of HLA-B*57:01 by KIR3DS1. *J Virol,* 89(10), 5213-21.

Oevermann, L., Michaelis, S. U., Mezger, M., Lang, P., Toporski, J., Bertaina, A., Zecca, M., Moretta, L., Locatelli, F. & Handgretinger, R. (2014) KIR B haplotype donors confer a reduced risk for relapse after haploidentical transplantation in children with ALL. *Blood,* 124(17), 2744-7.

Pando, M. J., Gardiner, C. M., Gleimer, M., McQueen, K. L. & Parham, P. (2003) The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. *The Journal of Immunology,* 171(12), 6640-9.

Parham, P. (2004) NK cells and trophoblasts: partners in pregnancy. *J Exp Med,* 200(8), 951-5.

Parham, P., Norman, P. J., Abi-Rached, L. & Guethlein, L. A. (2012) Human-specific evolution of killer cell immunoglobulin-like receptor recognition of major histocompatibility complex class I molecules. *Philos Trans R Soc Lond B Biol Sci,* 367(1590), 800-11.

Park, H., Rho, E. Y., In, J. W., Kim, I., Yoon, S. S., Park, S., Shin, S., Park, K. U. & Song, E. Y. (2015a) The impact of HLA and KIR ligand mismatching on unrelated allogeneic hematopoietic stem cell transplantation in Korean adult patients. *Ann Lab Med,* 35(1), 111-7.

Park, S., Kim, K., Jang, J. H., Kim, S. J., Kim, W. S., Kang, E. S. & Jung, C. W. (2015b) KIR alloreactivity based on the receptor-ligand model is associated with improved clinical outcomes of allogeneic hematopoietic stem cell transplantation: result of single center prospective study. *Hum Immunol,* 76(9), 636-43.

Pende, D., Falco, M., Vitale, M., Cantoni, C., Vitale, C., Munari, E., Bertaina, A., Moretta, F., Del Zotto, G., Pietra, G., Mingari, M. C., Locatelli, F. & Moretta, L. (2019) Killer Ig-Like Receptors (KIRs): Their Role in NK Cell Modulation and Developments Leading to Their Clinical Exploitation. *Front Immunol,* 10, 1179.

Petersdorf, E. W., Anasetti, C., Martin, P. J., Gooley, T., Radich, J., Malkki, M., Woolfrey, A., Smith, A., Mickelson, E. & Hansen, J. A. (2004) Limits of HLA mismatching in unrelated hematopoietic cell transplantation. *Blood,* 104(9), 2976-80.

Petersdorf, E. W., Gooley, T. A., Anasetti, C., Martin, P. J., Smith, A. G., Mickelson, E. M., Woolfrey, A. E. & Hansen, J. A. (1998) Optimizing Outcome After

Unrelated Marrow Transplantation by Comprehensive Matching of HLA Class I and II Alleles in the Donor and Recipient. *Blood,* 92(10), 3515-3520.

Pical-Izard, C., Crocchiolo, R., Granjeaud, S., Kochbati, E., Just-Landi, S., Chabannon, C., Frassati, C., Picard, C., Blaise, D., Olive, D. & Fauriat, C. (2015) Reconstitution of natural killer cells in HLA-matched HSCT after reduced-intensity conditioning: impact on clinical outcome. *Biol Blood Marrow Transplant,* 21(3), 429-39.

Pollard, M. O., Gurdasani, D., Mentzer, A. J., Porter, T. & Sandhu, M. S. (2018) Long reads: their purpose and place. *Hum Mol Genet,* 27(R2), R234-41.

Prakash, S., Sarangi, A. N., Alam, S., Sonawane, A., Sharma, R. K. & Agrawal, S. (2017) Putative role of KIR3DL1/3DS1 alleles and HLA-Bw4 ligands with end stage renal disease and long term renal allograft survival. *Gene,* 637, 219-229.

Pyo, C. W., Guethlein, L. A., Vu, Q., Wang, R., Abi-Rached, L., Norman, P. J., Marsh, S. G., Miller, J. S., Parham, P. & Geraghty, D. E. (2010) Different patterns of evolution in the centromeric and telomeric regions of group A and B haplotypes of the human killer cell Ig-like receptor locus. *PLoS One,* 5(12), e15115.

Pyo, C. W., Wang, R., Vu, Q., Cereb, N., Yang, S. Y., Duh, F. M., Wolinsky, S., Martin, M. P., Carrington, M. & Geraghty, D. E. (2013) Recombinant structures expand and contract inter and intragenic diversification at the KIR locus. *BMC Genomics,* 14, 89.

Rajagopalan, S. & Long, E. O. (1997) The direct binding of a p58 killer cell inhibitory receptor to human histocompatibility leukocyte antigen (HLA)-Cw4 exhibits peptide selectivity. *J Exp Med,* 185(8), 1523-8.

Rajagopalan, S. & Long, E. O. (2012) KIR2DL4 (CD158d): An activation receptor for HLA-G. *Front Immunol,* 3, 258.

Ribble, W., Kane, S. D. & Bullard, J. M. (2015) Long-range PCR amplification of DNA by DNA polymerase III holoenzyme from Thermus thermophilus. *Enzyme Res,* 2015, 837842.

Robinson, J., Barker, D. J., Georgiou, X., Cooper, M. A., Flicek, P. & Marsh, S. G. E. (2020) IPD-IMGT/HLA Database. *Nucleic Acids Res,* 48(D1), D948-D955.

Robinson, J., Halliwell, J. A., Hayhurst, J. D., Flicek, P., Parham, P. & Marsh, S. G. (2015) The IPD and IMGT/HLA database: allele variant databases. *Nucleic Acids Res,* 43(Database issue), D423-31.

Robinson, J., Mistry, K., McWilliam, H., Lopez, R. & Marsh, S. G. (2010) IPD - The Immuno Polymorphism Database. *Nucleic Acids Res,* 38(Database issue), D863-9.

Roe, D., Vierra-Green, C., Pyo, C. W., Eng, K., Hall, R., Kuang, R., Spellman, S., Ranade, S., Geraghty, D. E. & Maiers, M. (2017) Revealing complete complex KIR haplotypes phased by long-read sequencing technology. *Genes Immun,* 18(3), 127-34.

Rolstad, B. & Benestad, H. B. (1991) Spontaneous alloreactivity of natural killer (NK) and lymphokine-activated killer (LAK) cells from athymic rats against normal haemic cells. NK cells stimulate syngeneic but inhibit allogeneic haemopoiesis. *Immunology,* 74(1), 86-93.

Ruggeri, L., Capanni, M., Casucci, M., Volpi, I., Tosti, A., Perruccio, K., Urbani, E., Negrin, R. S., Martelli, M. F. & Velardi, A. (1999) Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood,* 94(1), 333-9.

Sahin, U., Dalva, K., Gungor, F., Ustun, C. & Beksac, M. (2018) Donor-recipient killer immunoglobulin like receptor (KIR) genotype matching has a protective effect on chronic graft versus host disease and relapse incidence following HLA-identical sibling hematopoietic stem cell transplantation. *Ann Hematol,* 97(6), 1027-39.

Sambrook, J. G., Bashirova, A., Andersen, H., Piatak, M., Vernikos, G. S., Coggill, P., Lifson, J. D., Carrington, M. & Beck, S. (2006) Identification of the ancestral killer immunoglobulin-like receptor gene in primates. *BMC Genomics,* 7, 209.

Santamaria, P., Reinsmoen, N. L., Lindstrom, A. L., Boyce-Jacino, M. T., Barbosa, J. J., Faras, A. J., McGlave, P. B. & Rich, S. S. (1994) Frequent HLA class I and DP sequence mismatches in serologically (HLA- A, HLA-B, HLA-DR) and molecularly (HLA-DRB1, HLA-DQA1, HLA-DQB1) HLA- identical unrelated bone marrow transplant pairs. *Blood,* 83(12), 3834-3834.

Santin, I., de Nanclares, G. P., Calvo, B., Gaafar, A., Castano, L., Group, G.-N. & Bilbao, J. R. (2006) Killer cell immunoglobulin-like receptor (KIR) genes in the Basque population: association study of KIR gene contents with type 1 diabetes mellitus. *Hum Immunol,* 67(1-2), 118-24.

Santourlidis, S., Trompeter, H. I., Weinhold, S., Eisermann, B., Meyer, K. L., Wernet, P. & Uhrberg, M. (2002) Crucial role of DNA methylation in determination of clonally distributed killer cell Ig-like receptor expression patterns in NK cells. *The Journal of Immunology,* 169(8), 4253-61.

Saulquin, X., Gastinel, L. N. & Vivier, E. (2003) Crystal structure of the human natural killer cell activating receptor KIR2DS2 (CD158j). *J Exp Med,* 197(7), 933-8.

Saunders, P. M., MacLachlan, B. J., Pymm, P., Illing, P. T., Deng, Y., Wong, S. C., Oates, C. V. L., Purcell, A. W., Rossjohn, J., Vivian, J. P. & Brooks, A. G. (2020) The molecular basis of how buried human leukocyte antigen polymorphism modulates natural killer cell function. *Proc Natl Acad Sci U S A,* 117(21), 11636-11647.

Saunders, P. M., Vivian, J. P., Baschuk, N., Beddoe, T., Widjaja, J., O'Connor, G. M., Hitchen, C., Pymm, P., Andrews, D. M., Gras, S., McVicar, D. W., Rossjohn, J. & Brooks, A. G. (2015) The interaction of KIR3DL1*001 with HLA class I molecules is dependent upon molecular microarchitecture within the Bw4 epitope. *J Immunol,* 194(2), 781-9.

Schadt, E. E., Turner, S. & Kasarskis, A. (2010) A window into third-generation sequencing. *Hum Mol Genet,* 19(R2), R227-40.

Schafer, J. L., Ries, M., Guha, N., Connole, M., Colantonio, A. D., Wiertz, E. J., Wilson, N. A., Kaur, A. & Evans, D. T. (2015) Suppression of a Natural Killer Cell Response by Simian Immunodeficiency Virus Peptides. *PLoS Pathog,* 11(9), e1005145.

Schetelig, J., Baldauf, H., Heidenreich, F., Massalski, C., Frank, S., Sauter, J., Stelljes, M., Ayuk, F. A., Bethge, W. A., Bug, G., Klein, S., Wendler, S., Lange, V., de Wreede, L. C., Furst, D., Kobbe, G., Ottinger, H. D., Beelen, D. W., Mytilineos, J., Fleischhauer, K., Schmidt, A. H. & Bornhauser, M. (2020) External validation of models for KIR2DS1/KIR3DL1-informed selection of hematopoietic cell donors fails. *Blood,* 135(16), 1386-1395.

Schmidt, A. H., Sauter, J., Baier, D. M., Daiss, J., Keller, A., Klussmeier, A., Mengling, T., Rall, G., Riethmuller, T., Schofl, G., Solloch, U. V., Torosian, T., Means, D., Kelly, H., Jagannathan, L., Paul, P., Giani, A. S., Hildebrand, S., Schumacher, S., Markert, J., Fussel, M., Hofmann, J. A., Schafer, T.,

Pingel, J., Lange, V. & Schetelig, J. (2020) Immunogenetics in stem cell donor registry work: The DKMS example (Part 2). *Int J Immunogenet,* 47(2), 139-148.

Selvakumar, A., Steffens, U. & Dupont, B. (1996) NK cell receptor gene of the KIR family with two IG domains but highest homology to KIR receptors with three IG domains. *Tissue Antigens,* 48(4 Pt 1), 285-94.

Sharma, D., Bastard, K., Guethlein, L. A., Norman, P. J., Yawata, N., Yawata, M., Pando, M., Thananchai, H., Dong, T., Rowland-Jones, S., Brodsky, F. M. & Parham, P. (2009) Dimorphic motifs in D0 and D1+D2 domains of killer cell Ig-like receptor 3DL1 combine to form receptors with high, moderate, and no avidity for the complex of a peptide derived from HIV and HLA-A*2402. *J Immunol,* 183(7), 4569-82.

Shaw, B. E., Logan, B. R., Spellman, S. R., Marsh, S. G. E., Robinson, J., Pidala, J., Hurley, C., Barker, J., Maiers, M., Dehn, J., Wang, H., Haagenson, M., Porter, D., Petersdorf, E. W., Woolfrey, A., Horowitz, M. M., Verneris, M., Hsu, K. C., Fleischhauer, K. & Lee, S. J. (2018) Development of an Unrelated Donor Selection Score Predictive of Survival after HCT: Donor Age Matters Most. *Biol Blood Marrow Transplant,* 24(5), 1049-1056.

Shaw, B. E., Mayor, N. P., Szydlo, R. M., Bultitude, W. P., Anthias, C., Kirkland, K., Perry, J., Clark, A., Mackinnon, S., Marks, D. I., Pagliuca, A., Potter, M. N., Russell, N. H., Thomson, K., Madrigal, J. A. & Marsh, S. G. E. (2017) Recipient/donor HLA and CMV matching in recipients of T-cell-depleted unrelated donor haematopoietic cell transplants. *Bone Marrow Transplant,* 52(5), 717-725.

Shimoni, A., Labopin, M., Lorentino, F., Van Lint, M. T., Koc, Y., Gulbas, Z., Tischer, J., Bruno, B., Blaise, D., Pioltelli, P., Afanasyev, B., Ciceri, F., Mohty, M. & Nagler, A. (2019) Killer cell immunoglobulin-like receptor ligand mismatching and outcome after haploidentical transplantation with post-transplant cyclophosphamide. *Leukemia,* 33(1), 230-239.

Shimoni, A., Vago, L., Bernardi, M., Yerushalmi, R., Peccatori, J., Greco, R., Shem-Tov, N., Lo Russo, A., Danylesko, I., Apel, A., Bonini, C., Lupo Stanghellini, M. T., Nagler, A. & Ciceri, F. (2017) Missing HLA C group 1 ligand in patients with AML and MDS is associated with reduced risk of relapse and better survival after allogeneic stem cell transplantation with fludarabine and treosulfan reduced toxicity conditioning. *Am J Hematol,* 92(10), 1011-1019.

Sim, M. J., Malaker, S. A., Khan, A., Stowell, J. M., Shabanowitz, J., Peterson, M. E., Rajagopalan, S., Hunt, D. F., Altmann, D. M., Long, E. O. & Boyton, R. J. (2017) Canonical and Cross-reactive Binding of NK Cell Inhibitory Receptors to HLA-C Allotypes Is Dictated by Peptides Bound to HLA-C. *Front Immunol,* 8, 193.

Sim, M. J. W., Rajagopalan, S., Altmann, D. M., Boyton, R. J., Sun, P. D. & Long, E. O. (2019) Human NK cell receptor KIR2DS4 detects a conserved bacterial epitope presented by HLA-C. *Proc Natl Acad Sci U S A,* 116(26), 12964-12973.

Sims, D., Sudbery, I., Ilott, N. E., Heger, A. & Ponting, C. P. (2014) Sequencing depth and coverage: key considerations in genomic analyses. *Nat Rev Genet,* 15(2), 121-32.

Single, R. M., Martin, M. P., Gao, X., Meyer, D., Yeager, M., Kidd, J. R., Kidd, K. K. & Carrington, M. (2007) Global diversity and evidence for coevolution of KIR and HLA. *Nat Genet,* 39(9), 1114-9.

Snyder, G. A., Brooks, A. G. & Sun, P. D. (1999) Crystal structure of the HLA-Cw3 allotype-specific killer cell inhibitory receptor KIR2DL2. *Proc Natl Acad Sci U S A,* 96(7), 3864-9.

Sobecks, R. M., Wang, T., Askar, M., Gallagher, M. M., Haagenson, M., Spellman, S., Fernandez-Vina, M., Malmberg, K. J., Muller, C., Battiwalla, M., Gajewski, J., Verneris, M. R., Ringden, O., Marino, S., Davies, S., Dehn, J., Bornhauser, M., Inamoto, Y., Woolfrey, A., Shaw, P., Pollack, M., Weisdorf, D., Millller, J., Hurley, C., Lee, S. J. & Hsu, K. (2015) Impact of KIR and HLA genotypes on outcomes after reduced-intensity conditioning hematopoietic cell transplantation. *Biol Blood Marrow Transplant,* 21(9), 1589-96.

Solloch, U. V., Schefzyk, D., Schafer, G., Massalski, C., Kohler, M., Pruschke, J., Heidl, A., Schetelig, J., Schmidt, A. H., Lange, V. & Sauter, J. (2020) Estimation of German KIR Allele Group Haplotype Frequencies. *Front Immunol,* 11, 429.

Stewart, C. A., Laugier-Anfossi, F., Vély, F., Saulquin, X., Riedmuller, J., Tisserant, A., Gauthier, L., Romagné, F., Ferracci, G., Arosa, F. A., Moretta, A., Sun, P. D., Ugolini, S. & Vivier, E. (2005) Recognition of peptide-MHC class I complexes by killer immunoglobulin-like receptors. *Proc Natl Acad Sci U S A,* 2005, 13224-9.

Symons, H. J., Leffell, M. S., Rossiter, N. D., Zahurak, M., Jones, R. J. & Fuchs, E. J. (2010) Improved survival with inhibitory killer immunoglobulin receptor (KIR) gene mismatches and KIR haplotype B donors after nonmyeloablative, HLA-haploidentical bone marrow transplantation. *Biol Blood Marrow Transplant,* 16(4), 533-42.

Taner, S. B., Pando, M. J., Roberts, A., Schellekens, J., Marsh, S. G., Malmberg, K. J., Parham, P. & Brodsky, F. M. (2011) Interactions of NK cell receptor KIR3DL1*004 with chaperones and conformation-specific antibody reveal a functional folded state as well as predominant intracellular retention. *J Immunol,* 186(1), 62-72.

Thiruchelvam-Kyle, L., Hoelsbrekken, S. E., Saether, P. C., Bjornsen, E. G., Pende, D., Fossum, S., Daws, M. R. & Dissen, E. (2017) The activating human NK cell receptor KIR2DS2 recognizes a beta2-microglobulin-independent ligand on cancer cells. *J Immunol,* 198(7), 2556-67.

Traherne, J. A., Martin, M., Ward, R., Ohashi, M., Pellett, F., Gladman, D., Middleton, D., Carrington, M. & Trowsdale, J. (2010) Mechanisms of copy number variation and hybrid gene formation in the KIR immune gene complex. *Hum Mol Genet,* 19(5), 737-51.

Trompeter, H. I., Gomez-Lozano, N., Santourlidis, S., Eisermann, B., Wernet, P., Vilches, C. & Uhrberg, M. (2005) Three structurally and functionally divergent kinds of promoters regulate expression of clonally distributed killer cell Ig-like receptors (KIR), of KIR2DL4, and of KIR3DL3. *The Journal of Immunology,* 174(7), 4135-43.

Trundley, A. E., Hiby, S. E., Chang, C., Sharkey, A. M., Santourlidis, S., Uhrberg, M., Trowsdale, J. & Moffett, A. (2006) Molecular characterization of KIR3DL3. *Immunogenetics,* 57(12), 904-16.

Tu, M. M., Mahmoud, A. B. & Makrigiannis, A. P. (2016) Licensed and Unlicensed NK Cells: Differential Roles in Cancer and Viral Control. *Front Immunol,* 7, 166.

Uhrberg, M. (2005) Shaping the human NK cell repertoire: an epigenetic glance at KIR gene regulation. *Mol Immunol,* 42(4), 471-5.

Uhrberg, M., Parham, P. & Wernet, P. (2002) Definition of gene content for nine common group B haplotypes of the Caucasoid population: KIR haplotypes contain between seven and eleven KIR genes. *Immunogenetics,* 54(4), 221-229.

Uhrberg, M., Valiante, N. M., Shum, B. P., Shilling, H., Lienert-Weidenbach, K., Corliss, B., Tyan, D., Lanier, L. & Parham, P. (1997) Human diversity in killer cell inhibitory receptor genes. *Immunity,* 7, 753-63.

Ullah, M. A., Hill, G. R. & Tey, S. K. (2016) Functional Reconstitution of Natural Killer Cells in Allogeneic Hematopoietic Stem Cell Transplantation. *Front Immunol,* 7, 144.

van Teijlingen, N. H., Holzemer, A., Korner, C., Garcia-Beltran, W. F., Schafer, J. L., Fadda, L., Suscovich, T. J., Brander, C., Carrington, M., Evans, D. T., van Baarle, D. & Altfeld, M. (2014) Sequence variations in HIV-1 p24 Gag-derived epitopes can alter binding of KIR2DL2 to HLA-C*03:04 and modulate primary natural killer cell function. *AIDS,* 28(10), 1399-408.

VandenBussche, C. J., Dakshanamurthy, S., Posch, P. E. & Hurley, C. K. (2006) A single polymorphism disrupts the killer Ig-like receptor 2DL2/2DL3 D1 domain. *J Immunol,* 177(8), 5347-57.

Vendelbosch, S., de Boer, M., Gouw, R. A., Ho, C. K., Geissler, J., Swelsen, W. T., Moorhouse, M. J., Lardy, N. M., Roos, D., van den Berg, T. K. & Kuijpers, T. W. (2013) Extensive variation in gene copy number at the killer immunoglobulin-like receptor locus in humans. *PLoS One,* 8(6), e67619.

Verneris, M. R., Miller, J. S., Hsu, K. C., Wang, T., Sees, J. A., Paczesny, S., Rangarajan, H., Lee, D. A., Spellman, S. R. & Lee, S. J. (2020) Investigation of donor KIR content and matching in children undergoing hematopoietic cell transplantation for acute leukemia. *Blood Adv,* 4(7), 1350-1356.

Vierra-Green, C., Roe, D., Hou, L., Hurley, C. K., Rajalingam, R., Reed, E., Lebedeva, T., Yu, N., Stewart, M., Noreen, H., Hollenbach, J. A., Guethlein, L. A., Wang, T., Spellman, S. & Maiers, M. (2012) Allele-level haplotype frequencies and pairwise linkage disequilibrium for 14 KIR loci in 506 European-American individuals. *PLoS ONE,* 7(11), e47491.

Vierra-Green, C., Roe, D., Jayaraman, J., Trowsdale, J., Traherne, J., Kuang, R., Spellman, S. & Maiers, M. (2016) Estimating KIR haplotype frequencies on a cohort of 10,000 individuals: A comprehensive study on population variations, typing resolutions, and reference haplotypes. *PLoS One,* 11(10), e0163973.

Vilches, C., Gardiner, C. M. & Parham, P. (2000a) Gene structure and promoter variation of expressed and nonexpressed variants of the KIR2DL5 gene. *J Immunol,* 165(11), 6416-21.

Vilches, C., Pando, M. J. & Parham, P. (2000b) Genes encoding human killer-cell Ig-like receptors with D1 and D2 extracellular domains all contain untranslated pseudoexons encoding a third Ig-like domain. *Immunogenetics,* 51(8-9), 639-46.

Vilches, C. & Parham, P. (2002) KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol,* 20, 217-51.

Vivian, J. P., Duncan, R. C., Berry, R., O'Connor, G. M., Reid, H. H., Beddoe, T., Gras, S., Saunders, P. M., Olshina, M. A., Widjaja, J. M., Harpur, C. M., Lin, J., Maloveste, S. M., Price, D. A., Lafont, B. A., McVicar, D. W., Clements, C. S., Brooks, A. G. & Rossjohn, J. (2011) Killer cell immunoglobulin-like receptor 3DL1-mediated recognition of human leukocyte antigen B. *Nature,* 479(7373), 401-5.

Wagner, I., Schefzyk, D., Pruschke, J., Schofl, G., Schone, B., Gruber, N., Lang, K., Hofmann, J., Gnahm, C., Heyn, B., Marin, W. M., Dandekar, R., Hollenbach, J. A., Schetelig, J., Pingel, J., Norman, P. J., Sauter, J., Schmidt, A. H. & Lange, V. (2018) Allele-level KIR genotyping of more than a million samples: workflow, algorithm, and observations. *Front Immunol,* 9(2843), 1-15.

Wang, H., Grzywacz, B., Sukovich, D., McCullar, V., Cao, Q., Lee, A. B., Blazar, B. R., Cornfield, D. N., Miller, J. S. & Verneris, M. R. (2007) The unexpected effect of cyclosporin A on CD56+CD16- and CD56+CD16+ natural killer cell subpopulations. *Blood,* 110(5), 1530-9.

Wauquier, N., Padilla, C., Becquart, P., Leroy, E. & Vieillard, V. (2010) Association of KIR2DS1 and KIR2DS3 with fatal outcome in Ebola virus infection. *Immunogenetics,* 62(11-12), 767-71.

Wauquier, N., Petitdemange, C., Tarantino, N., Maucourant, C., Coomber, M., Lungay, V., Bangura, J., Debre, P. & Vieillard, V. (2019) HLA-C-restricted viral epitopes are associated with an escape mechanism from KIR2DL2(+) NK cells in Lassa virus infection. *EBioMedicine,* 40, 605-613.

Weisdorf, D., Cooley, S., Wang, T., Trachtenberg, E., Haagenson, M. D., Vierra-Green, C., Spellman, S., Spahn, A., Vogel, J., Kobusingye, H., Fehninger, T., Woolfrey, A., Devine, S., Ross, M., Waller, E. K., Sobecks, R., Parham, P., Guethlein, L. A., Marsh, S. G. E., Miller, J. & participating center writing, c. (2019) KIR donor selection: Feasibility in identifying better donors. *Biol Blood Marrow Transplant,* 25(1), e28-32.

Weisdorf, D., Cooley, S., Wang, T., Trachtenberg, E., Vierra-Green, C., Spellman, S., Sees, J. A., Spahn, A., Vogel, J., Fehniger, T. A., Woolfrey, A. E., Devine, S. M., Ross, M., Waller, E. K., Sobecks, R. M., McGuirk, J., Oran, B., Farag, S. S., Shore, T., Van Besien, K., Marsh, S. G. E., Guethlein, L. A., Parham, P. & Miller, J. S. (2020) KIR B donors improve the outcome for AML patients given reduced intensity conditioning and unrelated donor transplantation. *Blood Adv,* 4(4), 740-754.

Willem, C., Makanga, D. R., Guillaume, T., Maniangou, B., Legrand, N., Gagne, K., Peterlin, P., Garnier, A., Bene, M. C., Cesbron, A., Le Bourgeois, A., Chevallier, P. & Retiere, C. (2019) Impact of KIR/HLA Incompatibilities on NK Cell Reconstitution and Clinical Outcome after T Cell-Replete Haploidentical Hematopoietic Stem Cell Transplantation with Posttransplant Cyclophosphamide. *J Immunol,* 202(7), 2141-2152.

Wilson, M. J., Torkar, M., Haude, A., Milne, S., Jones, T., Sheer, D., Beck, S. & Trowsdale, J. (2000) Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci U S A,* 97(9), 4778-83.

Winter, C. C., Gumperz, J. E., Parham, P., Long, E. O. & Wagtmann, N. (1998) Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *The Journal of Immunology,* 161, 571-77.

Winter, C. C. & Long, E. O. (1997) A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes. *J Immunol,* 158(9), 4026-4028.

Wroblewski, E. E., Parham, P. & Guethlein, L. A. (2019) Two to Tango: Co-evolution of Hominid Natural Killer Cell Receptors and MHC. *Front Immunol,* 10(177), 1-22.

Wu, S., Zeng, Y. J., Zhang, C., Deng, T. X., Xu, Y. Q. & Zhang, X. (2015) The role of the killer cell immunoglobulin-like receptor (KIR) "missing self" model in unrelated donor HSCT: a meta-analysis. *Transplant Proc,* 47(2)**,** 558-65.

Wu, X., Yao, Y., Bao, X., Zhou, H., Tang, X., Han, Y., Ma, X., Liu, Y., Chen, J., Zhou, H., Jing, S., Gu, B., Xu, Y., Sun, A., He, J. & Wu, D. (2016) KIR2DS4 and its variant KIR1D are associated with acute graft-versus-host disease, cytomegalovirus, and overall survival after sibling-related HLA-matched transplantation in patients with donors with KIR gene haplotype A. *Biol Blood Marrow Transplant,* 22(2)**,** 220-5.

Yahng, S. A., Jeon, Y. W., Yoon, J. H., Shin, S. H., Lee, S. E., Cho, B. S., Eom, K. S., Kim, Y. J., Lee, S., Min, C. K., Cho, S. G., Kim, D. W., Lee, J. W., Min, W. S. & Kim, H. J. (2016) Negative impact of unidirectional host-versus-graft killer cell immunoglobulin-like receptor ligand mismatch on transplantation outcomes after unmanipulated haploidentical peripheral blood stem cell transplantation for acute myeloid leukemia. *Biol Blood Marrow Transplant,* 22(2)**,** 316-23.

Zhou, H., Bao, X., Wu, X., Tang, X., Wang, M., Wu, D. & He, J. (2014) Donor selection for KIR B haplotype of the centromeric motifs can improve the outcome after HLA-identical sibling hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant,* 20**,** 98-105.

Appendices

Appendix A – Additional Figures and Tables

Appendix A.i Chapter 3 – Additional Tables & Figures

Raw KIR Allelic Carrier Frequencies

KIR2DL1* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype		KIR2DL1* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype	
	Freq.	n	Freq.	n	Freq.	n		Freq.	n	Freq.	n	Freq.	n
00302	0.618	165	0.756	65	0.552	100	**007**	0.007	3	0.000	0	0.017	3
00201	0.416	111	0.012	51	0.348	60	**034**	0.004	2	0.023	2	0.000	0
00401	0.240	64	0.081	1	0.033	63	**01101**	0.004	1	0.000	0	0.006	1
00101	0.049	13	0.012	7	0.022	6	**020**	0.011	1	0.000	0	0.006	1
032N	0.019	5	0.012	1	0.017	4	**Negative**	0.032	9	0.000	0	0.471	9
008	0.015	4	0.035	1	0.006	3	***Failed***	-	*14*	-	*4*	-	*10*
Novel	0.015	4	0.756	3	0.552	1							

Table 30 – Carrier frequencies of KIR2DL1 for total cohort (n=281), KIR A/A diplotype individuals (n=90), and KIR B/x diplotype individuals (n=191).

The number of individuals negative for KIR2DL1 (verified by presence/absence testing) and failed tests (positive by presence/absence testing, failed gene sequencing) are also listed.

KIR2DL2* Allele	Total Cohort		KIR B/x Diplotype	
	Freq.	n	Freq.	n
00101	0.278	78	0.408	78
00301	0.246	69	0.361	69
00602	0.007	2	0.010	2
Negative	0.498	140	0.262	50
Failed	*N/A*	*0*	*N/A*	*0*

Table 31 - Carrier frequencies of KIR2DL2 for total cohort (n=281), KIR A/A diplotype individuals (n=90), and KIR B/x diplotype individuals (n=191).

The number of individuals negative for KIR2DL2 (verified by presence/absence testing) and failed tests (positive by presence/absence testing, failed gene sequencing) are also listed.

KIR2DL3* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype		KIR2DL3* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype	
	Freq.	n	Freq.	n	Freq.	n		Freq.	n	Freq.	n	Freq.	n
00101	0.612	170	0.733	66	0.553	104	**006**	0.004	1	0.011	1	0.000	0
00201	0.406	113	0.567	51	0.330	62	**013**	0.004	1	0.011	1	0.000	0
00501	0.054	15	0.089	8	0.037	7	**015**	0.004	1	0.011	1	0.000	0
003	0.040	11	0.044	4	0.037	7	**Novel**	0.004	1	0.011	1	0.000	0
00110	0.014	4	0.011	1	0.016	3	**Negative**	0.082	23	0.000	0	0.120	23
01202	0.007	2	0.011	1	0.005	1	***Failed***	-	*3*	-	*0*	-	*3*

Table 32 – Carrier frequencies of KIR2DL3 for total cohort (n=281), KIR A/A diplotype individuals (n=90), and KIR B/x diplotype individuals (n=191).

The number of individuals negative for KIR2DL3 (verified by presence/absence testing) and failed tests (positive by presence/absence testing, failed gene sequencing) are also listed.

KIR2DL4* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype		KIR2DL4* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype	
	Freq.	n	Freq.	n	Freq.	n		Freq.	n	Freq.	n	Freq.	n
00102	0.355	99	0.300	27	0.381	72	**Novel**	0.022	6	0.044	4	0.011	2
00501	0.344	96	0.033	3	0.492	93	**00902**	0.004	1	0.011	1	0.000	0
00801	0.333	93	0.422	38	0.291	55	**010**	0.004	1	0.000	0	0.005	1
00802	0.294	82	0.422	38	0.233	44	**013**	0.004	1	0.011	1	0.000	0
01101	0.287	80	0.311	28	0.275	52	**024**	0.004	1	0.011	1	0.000	0
00103	0.143	40	0.178	16	0.127	24	**Negative**	0.000	0	0.000	0	0.000	0
00602	0.057	16	0.067	6	0.053	10	***Failed***	N/A	*2*	N/A	*0*	N/A	*2*

Table 33 – Carrier frequencies of KIR2DL4 for total cohort (n=281), KIR A/A diplotype individuals (n=90), and KIR B/x diplotype individuals (n=191).

The number of individuals negative for KIR2DL4 (verified by presence/absence testing) and failed tests (positive by presence/absence testing, failed gene sequencing) are also listed.

KIR3DL1S1* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype		KIR3DL1S1* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype	
	Freq.	n	Freq.	n	Freq.	n		Freq.	n	Freq.	n	Freq.	n
KIR3DS1*01301	0.319	88	0.000	0	0.473	88	**KIR3DL1*009**	0.014	4	0.000	0	0.021	4
KIR3DL1*00101	0.313	87	0.411	37	0.266	50	**KIR3DL1*052**	0.014	4	0.000	0	0.021	4
KIR3DL1*00501	0.277	77	0.322	29	0.255	48	**KIR3DL1*053**	0.014	4	0.011	1	0.016	3
KIR3DL1*00401	0.255	71	0.389	35	0.191	36	**KIR3DL1*01501**	0.004	1	0.011	1	0.000	0
KIR3DL1*002	0.227	63	0.167	15	0.255	48	**KIR3DL1*01701**	0.004	1	0.000	0	0.005	1
KIR3DL1*01502	0.140	39	0.144	13	0.138	26	**KIR3DL1*021**	0.004	1	0.000	0	0.005	1
KIR3DL1*008	0.083	23	0.144	13	0.053	10	**KIR3DL1*033**	0.004	1	0.000	0	0.005	1
KIR3DL1*00701	0.058	16	0.067	6	0.053	10	**KIR3DL1*113**	0.004	1	0.011	1	0.000	0
KIR3DL1*02001	0.047	13	0.044	4	0.048	9	**KIR3DS1 Novel**	0.004	1	0.000	0	0.005	1
KIR3DL1*00402	0.036	10	0.033	3	0.037	7	***KIR3DL1 Negative***	*0.029*	*8*	*0.000*	*0*	*0.043*	*8*
KIR3DL1*019	0.022	6	0.044	4	0.011	2	***KIR3DL1 Failed***	*N/A*	*3*	*N/A*	*0*	*N/A*	*3*
KIR3DS1*049N	0.022	6	0.000	0	0.032	6	***KIR3DS1 Negative***	*0.659*	*182*	*1.000*	*90*	*0.495*	*92*
KIR3DL1 Novel	0.022	6	0.011	1	0.027	5	***KIR3DS1 Failed***	*N/A*	*5*	*N/A*	*0*	*N/A*	*5*

Table 34 – Carrier frequencies of KIR3DL1S1 for total cohort (n=281), KIR A/A diplotype individuals (n=90), and KIR B/x diplotype individuals (n=191).

The number of individuals negative for KIR3DL1 or KIR3DS1 (verified by presence/absence testing) and failed tests (positive by presence/absence testing, failed gene sequencing) are also listed. KIR3DS1 alleles are shaded for clarity. No individual was negative for both KIR3DL1 and KIR3DS1.

KIR3DL2* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype		KIR3DL2* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype	
	Freq.	n	Freq.	n	Freq.	n		Freq.	n	Freq.	n	Freq.	n
00201	0.343	92	0.302	26	0.362	66	**01004**	0.007	2	0.012	1	0.005	1
00701	0.306	82	0.000	0	0.449	82	**023**	0.007	2	0.012	1	0.005	1
00101	0.262	71	0.372	32	0.211	39	**10701**	0.007	2	0.000	0	0.011	2
00103	0.214	58	0.256	22	0.195	37	**00602**	0.004	1	0.012	1	0.000	0
00301	0.170	47	0.221	19	0.146	28	**01102**	0.004	1	0.012	1	0.000	0
00901	0.118	32	0.186	16	0.086	16	**01301**	0.004	1	0.012	1	0.000	0
00501	0.096	26	0.140	12	0.076	14	**016**	0.004	1	0.012	1	0.000	0
01001	0.077	21	0.070	6	0.081	15	**01901**	0.004	1	0.000	0	0.005	1
008	0.059	16	0.058	5	0.059	11	**01902**	0.004	1	0.000	0	0.005	1
01101	0.052	14	0.035	3	0.059	11	**048**	0.004	1	0.012	1	0.000	0
00902	0.044	12	0.047	4	0.043	8	**06002**	0.004	1	0.012	1	0.000	0
018	0.030	8	0.000	0	0.043	8	**06202**	0.004	1	0.012	1	0.000	0
Novel	0.018	7	0.035	3	0.011	4	**076**	0.004	1	0.000	0	0.005	1
00709	0.015	4	0.012	1	0.016	3	**Negative**	0.000	0	0.000	0	0.000	0
00601	0.011	3	0.012	1	0.011	2	***Failed***	*N/A*	*10*	*N/A*	*4*	*N/A*	*6*
020	0.011	3	0.023	2	0.005	1							

Table 35 – Carrier frequencies of KIR3DL2 for total cohort (n=281), KIR A/A diplotype individuals (n=90), and KIR B/x diplotype individuals (n=191).

The number of individuals negative for KIR3DL1 (verified by presence/absence testing) and failed tests (positive by presence/absence testing, failed gene sequencing) are also listed.

KIR3DL3* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype		KIR3DL3* Allele	Total Cohort		KIR A/A Diplotype		KIR B/x Diplotype	
	Freq.	n	Freq.	n	Freq.	n		Freq.	n	Freq.	n	Freq.	n
00101	0.250	70	0.367	33	0.195	37	**01702**	0.014	4	0.022	2	0.011	2
00301	0.236	66	0.000	0	0.347	66	**02501**	0.014	4	0.022	2	0.011	2
00901	0.168	47	0.133	12	0.184	35	**036**	0.014	4	0.000	0	0.021	4
01402	0.154	43	0.000	0	0.226	43	**01307**	0.011	3	0.033	3	0.000	0
01701	0.079	22	0.111	10	0.063	12	**01309**	0.011	3	0.033	3	0.000	0
00206	0.079	22	0.100	9	0.068	13	**01601**	0.011	3	0.000	0	0.016	3
01302	0.071	20	0.189	17	0.016	3	**020**	0.014	4	0.011	1	0.016	3
00202	0.064	18	0.111	10	0.042	8	**031**	0.011	3	0.022	2	0.005	1
00102	0.061	17	0.089	8	0.047	9	**00209**	0.011	3	0.022	2	0.005	1
00601	0.057	16	0.067	6	0.053	10	**00904**	0.007	2	0.000	0	0.011	2
00602	0.043	12	0.044	4	0.042	8	**01101**	0.007	2	0.022	2	0.000	0
007	0.043	12	0.000	0	0.063	12	**012**	0.007	2	0.022	2	0.000	0
00801	0.039	11	0.100	9	0.011	2	**01406**	0.007	2	0.000	0	0.011	2
00402	0.036	10	0.000	0	0.053	10	**02701**	0.007	2	0.022	2	0.000	0
01001	0.036	10	0.022	2	0.042	8	**00104**	0.004	1	0.011	1	0.000	0
01501	0.036	10	0.044	4	0.032	6	**00205**	0.004	1	0.011	1	0.000	0
01002	0.032	9	0.033	3	0.032	6	**00302**	0.004	1	0.000	0	0.005	1
Novel	0.029	8	0.011	1	0.037	7	**01105**	0.004	1	0.011	1	0.000	0
00207	0.025	7	0.033	3	0.021	4	**01404**	0.004	1	0.000	0	0.005	1
01403	0.025	7	0.000	0	0.037	7	**02601**	0.004	1	0.000	0	0.005	1
019	0.025	7	0.011	1	0.032	6	**02703**	0.004	1	0.000	0	0.005	1
00103	0.021	6	0.000	0	0.032	6	**02705**	0.004	1	0.000	0	0.005	1
00201	0.021	6	0.033	3	0.016	3	**030**	0.004	1	0.011	1	0.000	0
00902	0.021	6	0.011	1	0.026	5	**044**	0.004	1	0.011	1	0.000	0
01303	0.021	6	0.022	2	0.021	4	**048**	0.004	1	0.000	0	0.005	1
01405	0.021	6	0.000	0	0.032	6	**053**	0.004	1	0.011	1	0.000	0
041	0.018	5	0.022	2	0.016	3	**057**	0.004	1	0.000	0	0.005	1
00802	0.018	5	0.022	2	0.016	3	**Negative**	0.000	0	0.000	0	0.000	0
01102	0.018	5	0.022	2	0.016	3	***Failed***	*N/A*	*1*	*N/A*	*0*	*N/A*	*1*

Table 36 – Carrier frequencies of KIR3DL3 for total cohort (n=281), KIR A/A diplotype individuals (n=90), and KIR B/x diplotype individuals (n=191).

The number of individuals negative for KIR3DL3 (verified by presence/absence testing) and failed tests (positive by presence/absence testing, failed gene sequencing) are also listed.

Appendix A.ii Chapter 4 – Additional Tables & Figures

		Cohort	
Model	Category	Cohort A (All Transplants) n *(%)*	Cohort B (T cell depleted RIC Transplants) n *(%)*
KIR B Content Scoring Model 2010 (Donor)	Neutral	82 *(71.3%)*	62 *(70.5%)*
	Better	23 *(20.0%)*	19 *(21.6%)*
	Best	10 *(8.7%)*	7 (8.0%)
KIR Receptor/Ligand Mismatching Model (Donor KIR/Recipient HLA)	Matched	38 *(33.0%)*	30 *(34.1%)*
	Mismatched	77 *(67.0%)*	58 *(65.9%)*
Donor KIR Diplotype Characteristics	A/A	34 *(29.6%)*	23 *(26.1%)*
	B/x	81 *(70.4%)*	65 *(73.9%)*
Recipient C1/C2	C1/C1	51 *(44.3%)*	40 *(45.5%)*
	C1/C2	48 *(41.7%)*	38 *(43.2%)*
	C2/C2	16 *(13.9%)*	10 *(11.4%)*
Donor KIR2DS1	Negative	76 *(66.1%)*	56 *(63.6%)*
	Positive	39 *(33.9%)*	32 *(36.4%)*

Table 37 – KIR Characteristics of cohort transplant pairs (n=115).

Characteristics were comparable in both cohorts investigated.

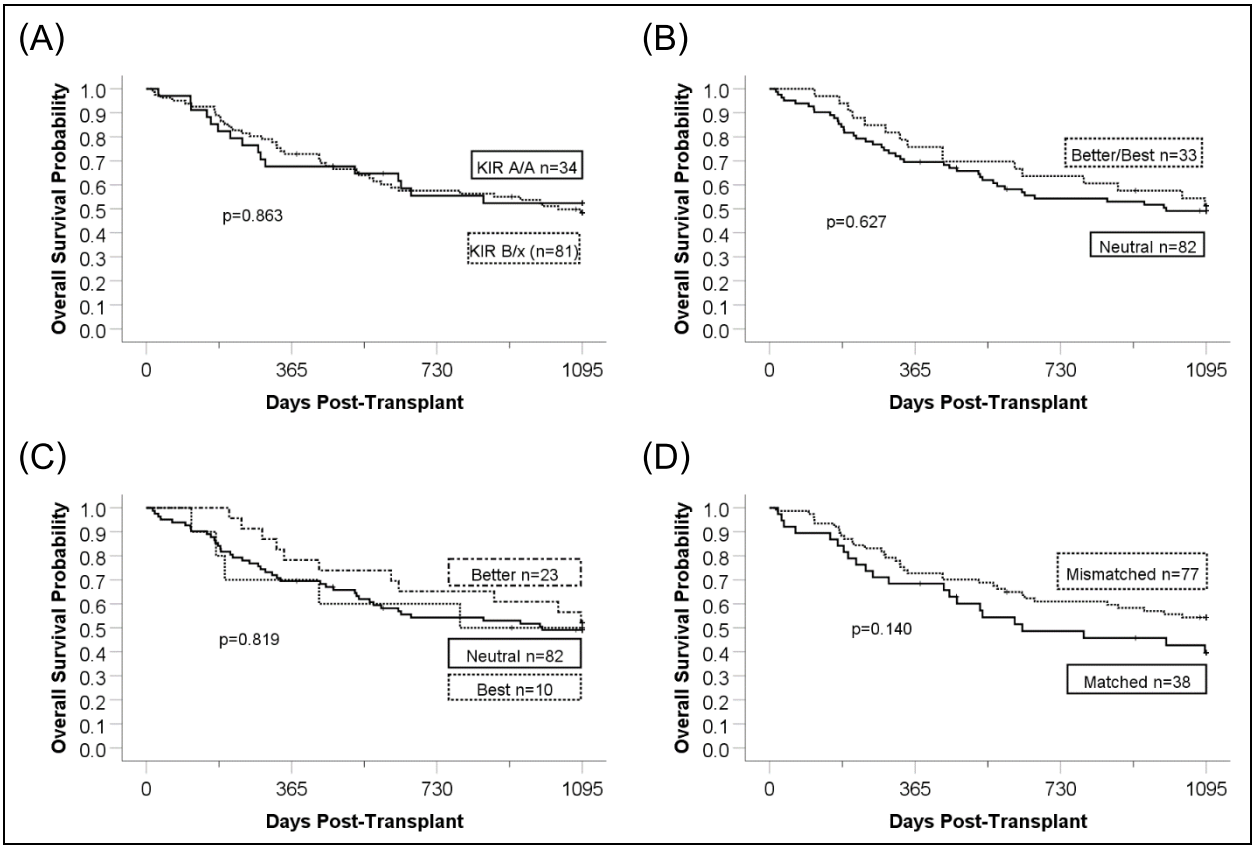


Figure 38 – Comparison of Overall Survival (OS) between investigated KIR assessment models in Cohort A (n=115).

(A) Donor KIR A/A versus B/x diplotypes (p=0.863), (B) KIR B Content Scoring Model (grouping Better and Best (p=0.627) (Cooley et al., 2010), (C) B Content Scoring Model (0.819) (Cooley et al., 2010), (D) KIR Receptor/Ligand Mismatching Model (p=0.140) (Leung et al., 2004).

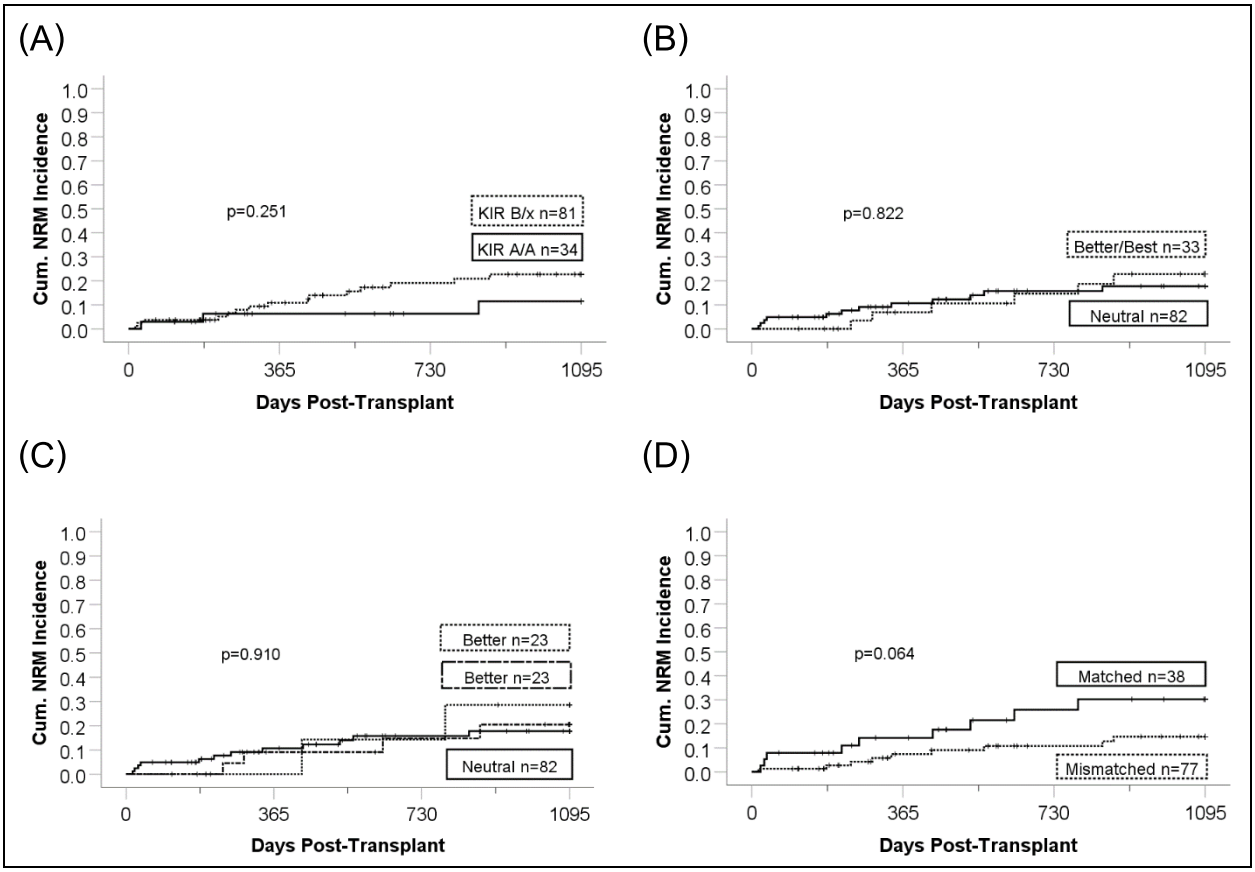


Figure 39 – Comparison of non-relapse mortality (NRM) between investigated KIR assessment models in Cohort A (n=115).

(A) Donor A/A versus B/x (p=0.251), (B) KIR B Content Scoring Model (grouping Better and Best) (Cooley et al., 2010) (p=0.822), (C) B Content Scoring Model (p=0.910) (Cooley et al., 2010), (D) KIR Receptor/Ligand Mismatching Model (p=0.064, p_c=0.256) (Leung et al., 2004).

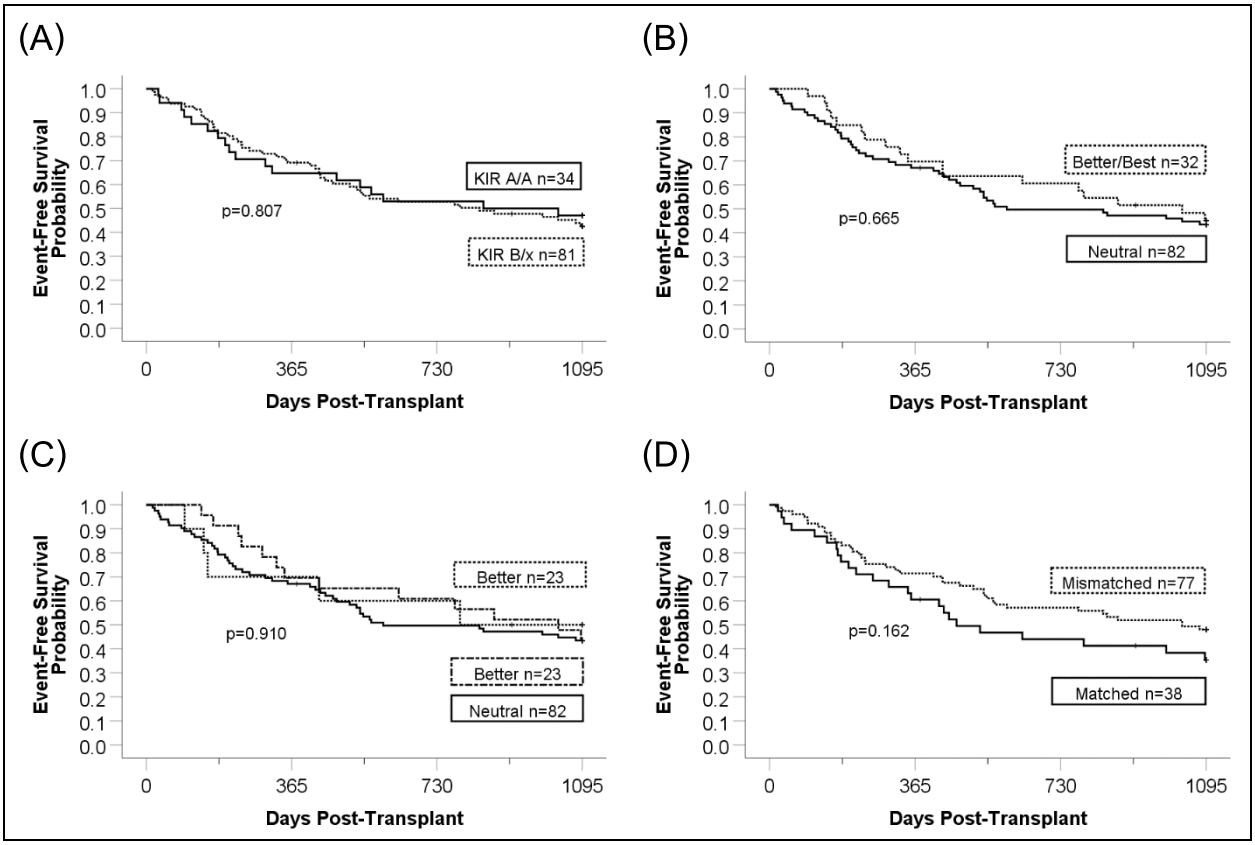


Figure 40 – Comparison of EFS between investigated KIR assessment models in Cohort A (n=115).

(A) Donor KIR A/A versus B/x (p=0.807), (B) KIR B Content Scoring Model (grouping Better and Best) (Cooley et al., 2010) (p=0.665), (C) B Content Scoring Model (Cooley et al., 2010) (p=0.910), (D) KIR Receptor/Ligand Mismatching Model (p=0.162) (Leung et al., 2004).

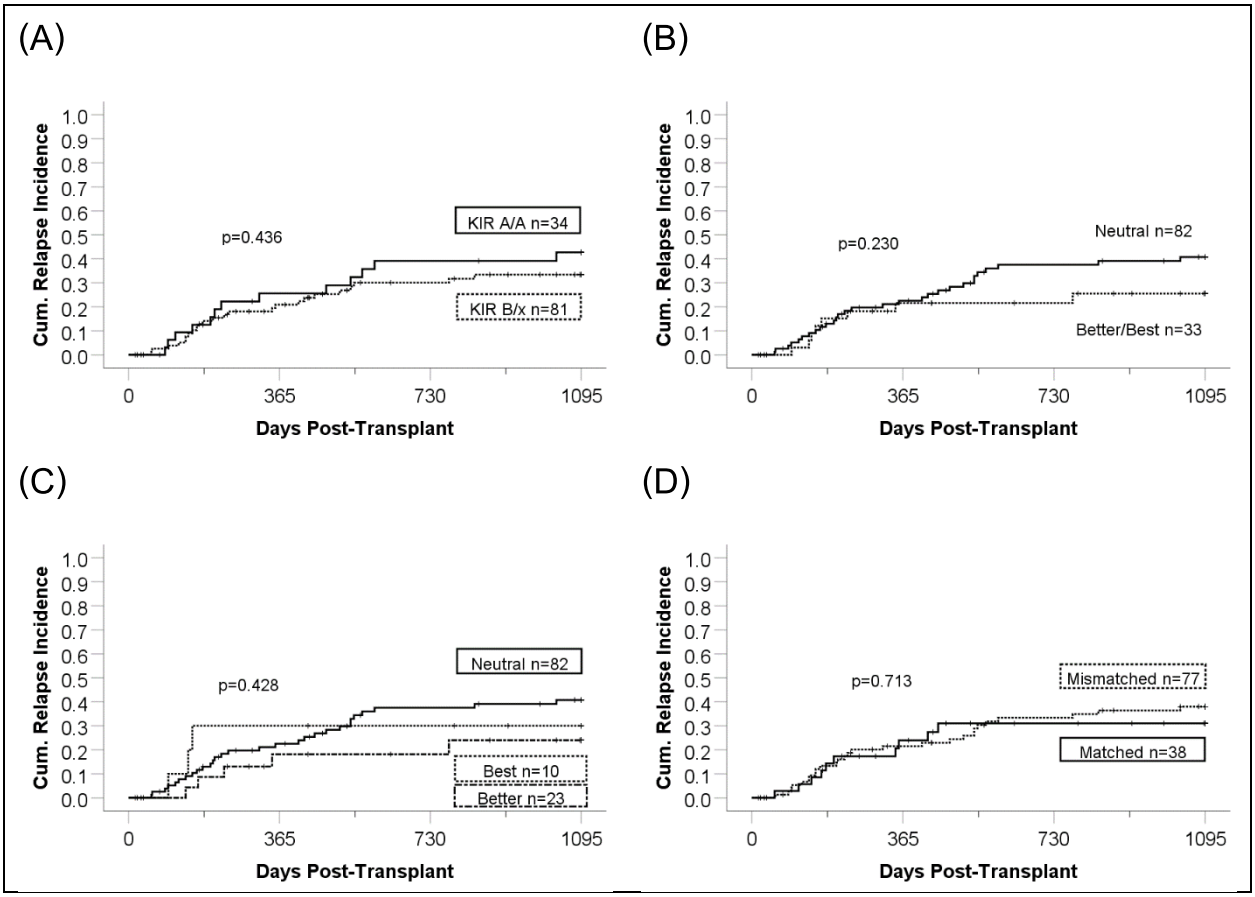


Figure 41 – Comparison of relapse between investigated KIR assessment models in Cohort A (n=115).

(A) Donor KIR A/A versus B/x (p=0.436), (B) KIR B Content Scoring Model (grouping Better and Best) (Cooley et al., 2010) (p=0.230), (C) B Content Scoring Model (Cooley et al., 2010) (p=0.246), (D) KIR Receptor/Ligand Mismatching Model (p=0.713) (Leung et al., 2004).

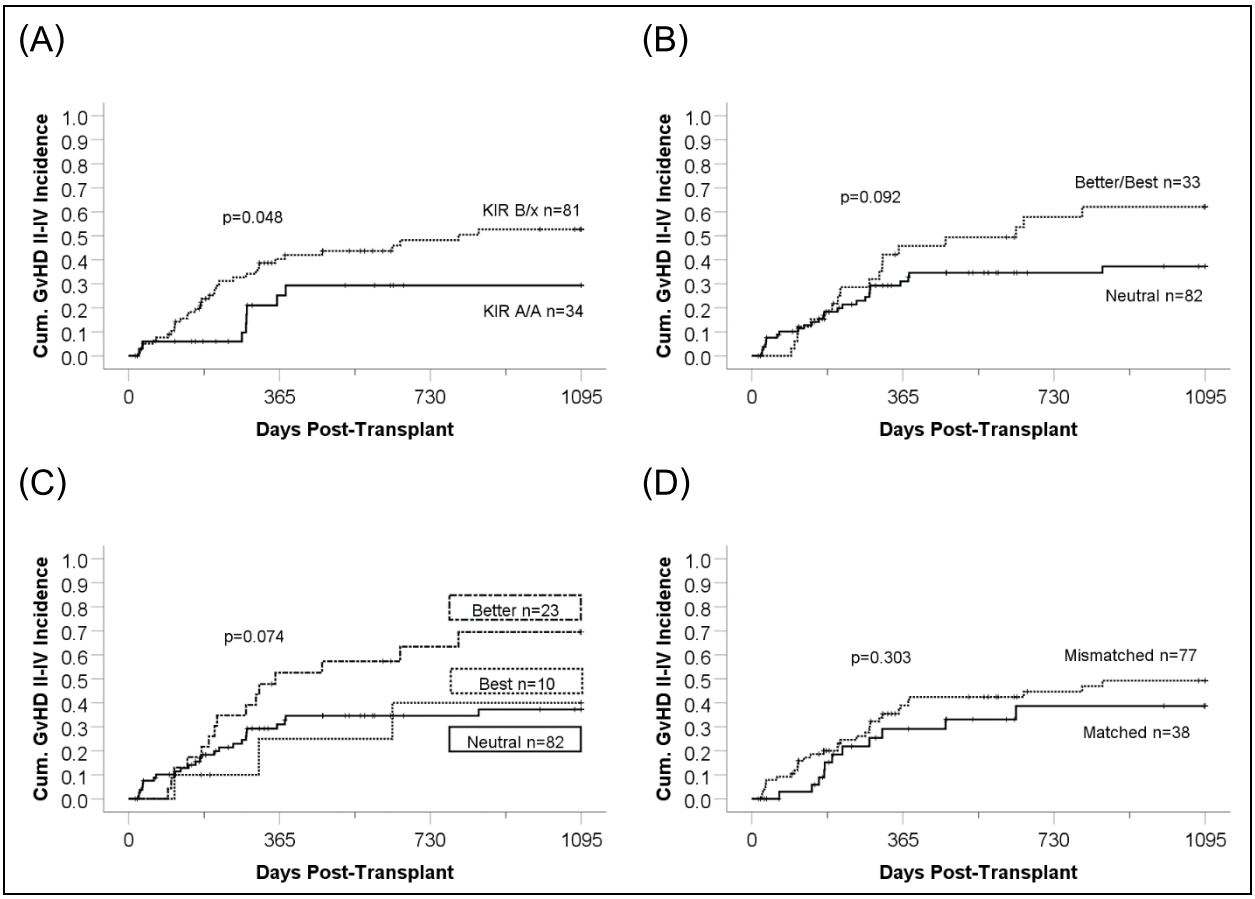


Figure 42 – Comparison of GvHD grade II-IV between investigated KIR assessment models in Cohort A (n=115).

(A) Donor KIR A/A versus B/x (p=0.048, p_c=0.192), (B) KIR B Content Scoring Model (grouping Better and Best) (p=0.092, p_c=0.222) (Cooley et al., 2009), (C) B Content Scoring Model (p=0.074, p_c=0.222) (Cooley et al., 2010), (D) KIR Receptor/Ligand Mismatching Model (p=0.303) (Leung et al., 2004).

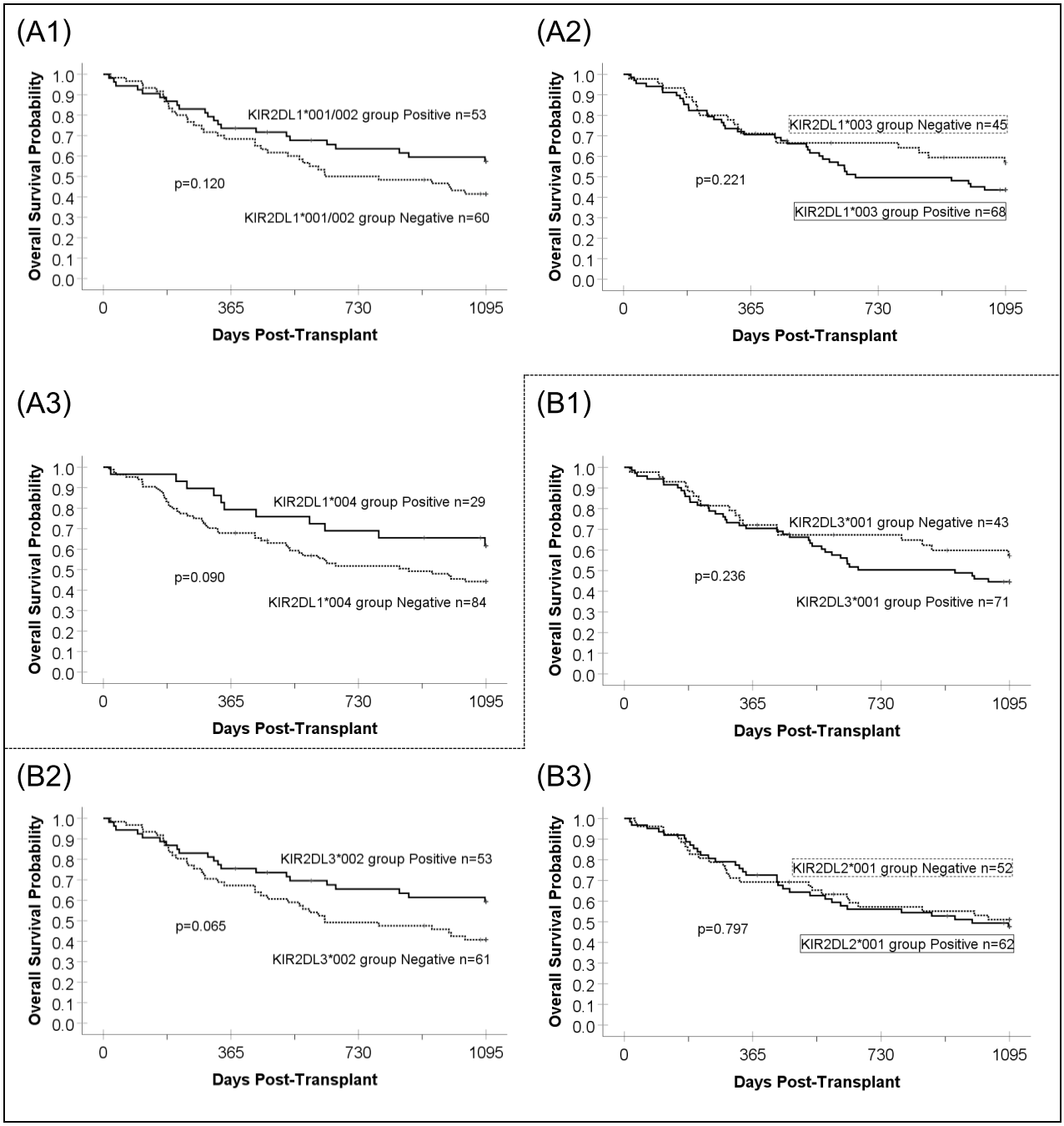


Figure 43 – The impact of donor KIR2DL1 (n=113) and KIR2DL2/3 (n=114) allele group presence/absence upon 3-year post-transplant OS in Cohort A.

(A) Transplants using KIR2DL1*001/002 group positive donors displayed a weak trend towards improving EFS (p=0.120). (A2) KIR2DL1*003 group presence/absence in the donor did not influence EFS (p=0.221). (A3) KIR2DL1*004 group positive donors trended towards improving EFS (p=0.090, p_c=0.450). (B1) KIR2DL1*003 group presence/absence in the donor did not influence EFS (p=0.221). (B2) KIR2DL1*004 group positive donors trended towards improving EFS (p=0.065, p_c=0.390). (B3) KIR2DL2*001 presence/absence in the donor did not influence 3-year post-transplant EFS (p=0.797).

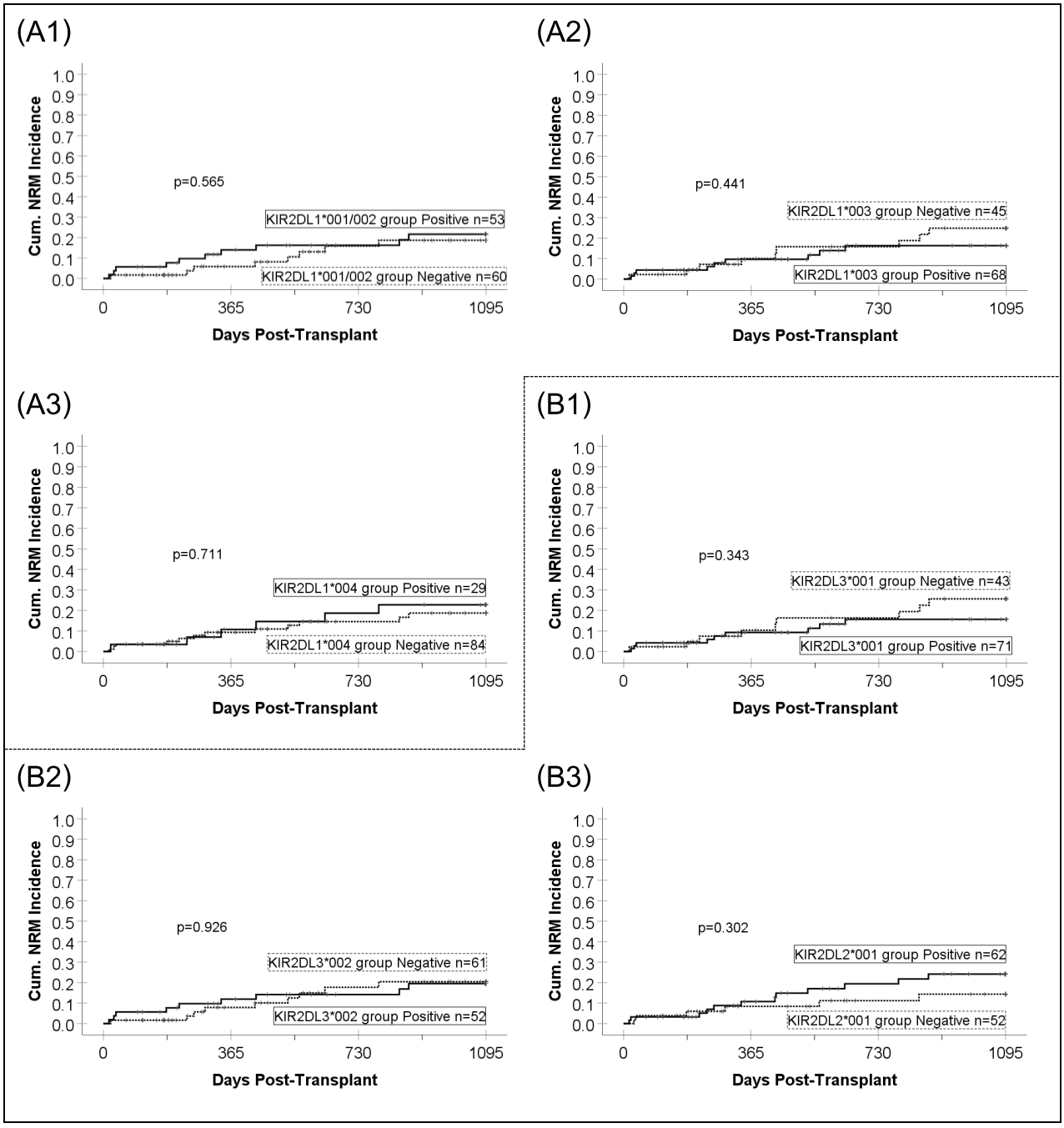


Figure 44 – The impact of donor KIR2DL1 (n=113) and KIR2DL2/3 (n=114) allele group presence/absence upon 3-year post-transplant NRM in Cohort A.

No KIR2DL1/2/3 allele groups investigated influenced 3-year NRM incidence. (A1) KIR2DL1*001/002 group donor presence/absence (p=0.565). (A2) KIR2DL1*003 group donor presence/absence (p=0.441). (A3) KIR2DL1*004 group donor presence/absence (p=0.711). (B1) KIR2DL3*001 group donor presence/absence (p=0.343). (B2) KIR2DL3*002 group donor presence/absence (p=0.926). (B3) KIR2DL2*001 group donor presence/absence (p=0.302).

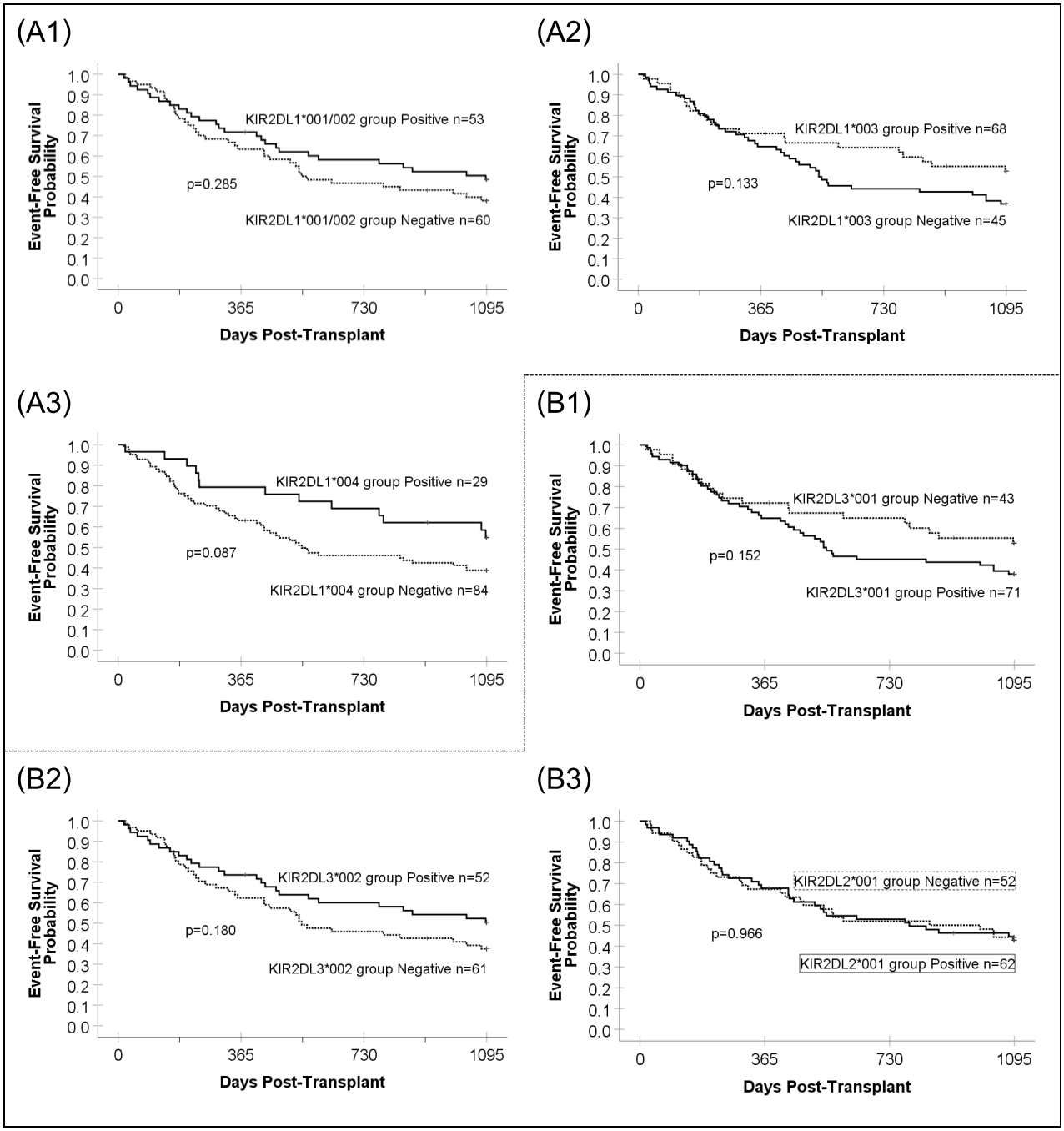


Figure 45 – The impact of donor KIR2DL1 (n=113) and KIR2DL2/3 (n=114) allele group presence/absence upon 3-year post-transplant EFS in Cohort A.

(A1) KIR2DL1*001 group presence/absence did not influence 3-year EFS (p=0.285). (A2) KIR2DL1*003 group positive donors displayed a weak trend towards decreasing EFS (p=0.133). (A3) KIR2DL1*004 group positive donors trended towards improving EFS (p=0.087, p_c=0.522). (B1) KIR2DL3*001 group positive donors displayed a weak trend towards decreasing EFS (p=0.152). (B2) KIR2DL3*002 group positive donors displayed a very weak trend towards improving EFS (p=0.180). (B3) KIR2DL2*001 presence/absence in the donor did not influence 3-year EFS (p=0.966).

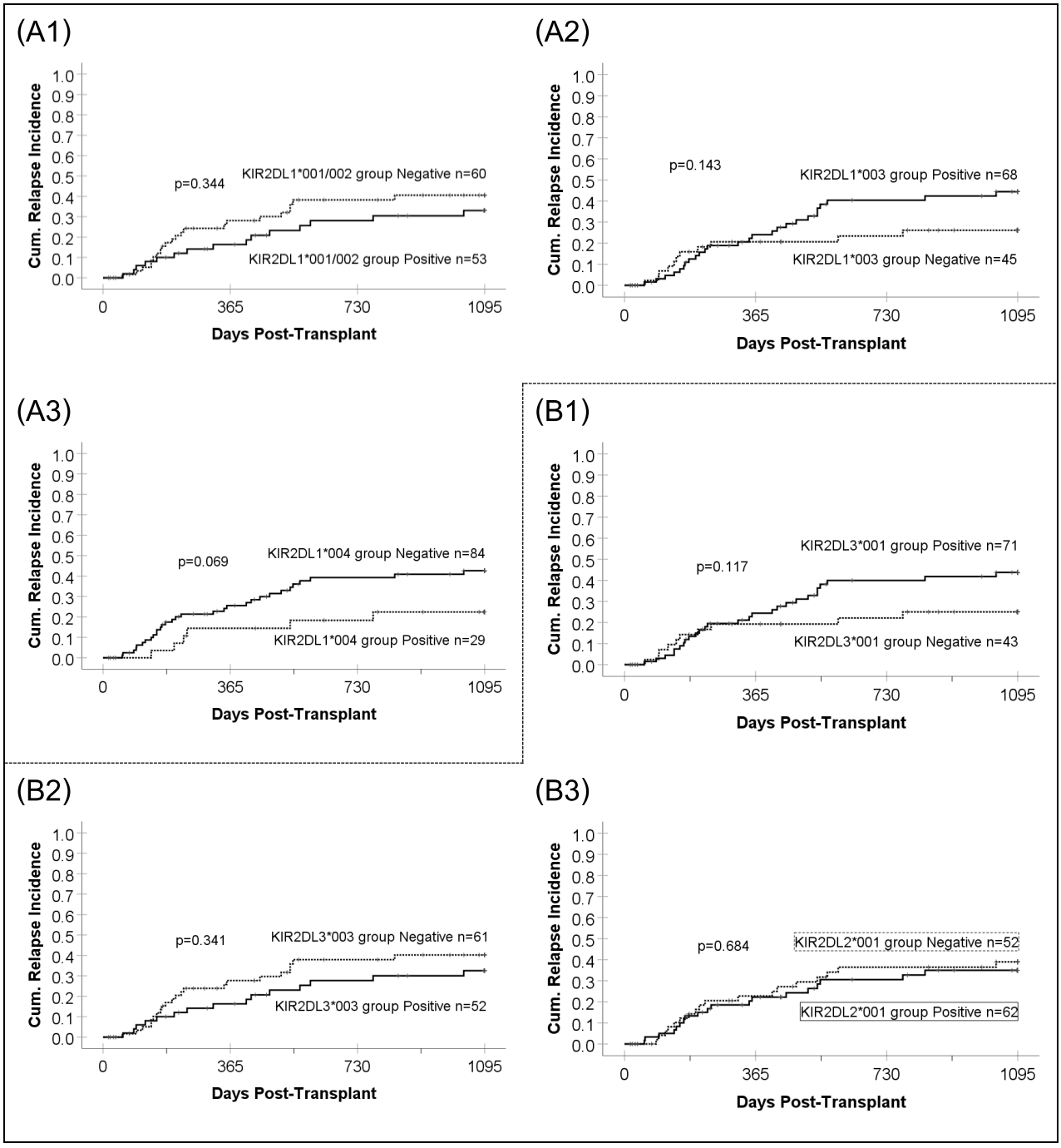


Figure 46 – The impact of donor KIR2DL1 (n=113) and KIR2DL2/3 (n=114) allele group presence/absence upon 3-year post-transplant relapse incidence in Cohort A.

(A1) KIR2DL1*001 group presence/absence did not influence 3-year relapse incidence (p=0.344). (A2) KIR2DL1*003 group positive donors displayed a weak trend towards increasing relapse incidence (p=0.143). (A3) KIR2DL1*004 group positive donors trended towards reducing relapse incidence (p=0.069, p_c=0.414). (B1) Transplants using KIR2DL3*001 group positive donors trended towards increasing 3-year relapse incidence (p=0.117). (B2) KIR2DL3*002 group and (B3) KIR2DL2*001 group presence/absence in the donor did not influence 3-year relapse incidence (p=0.341 and p=0.684 respectively).

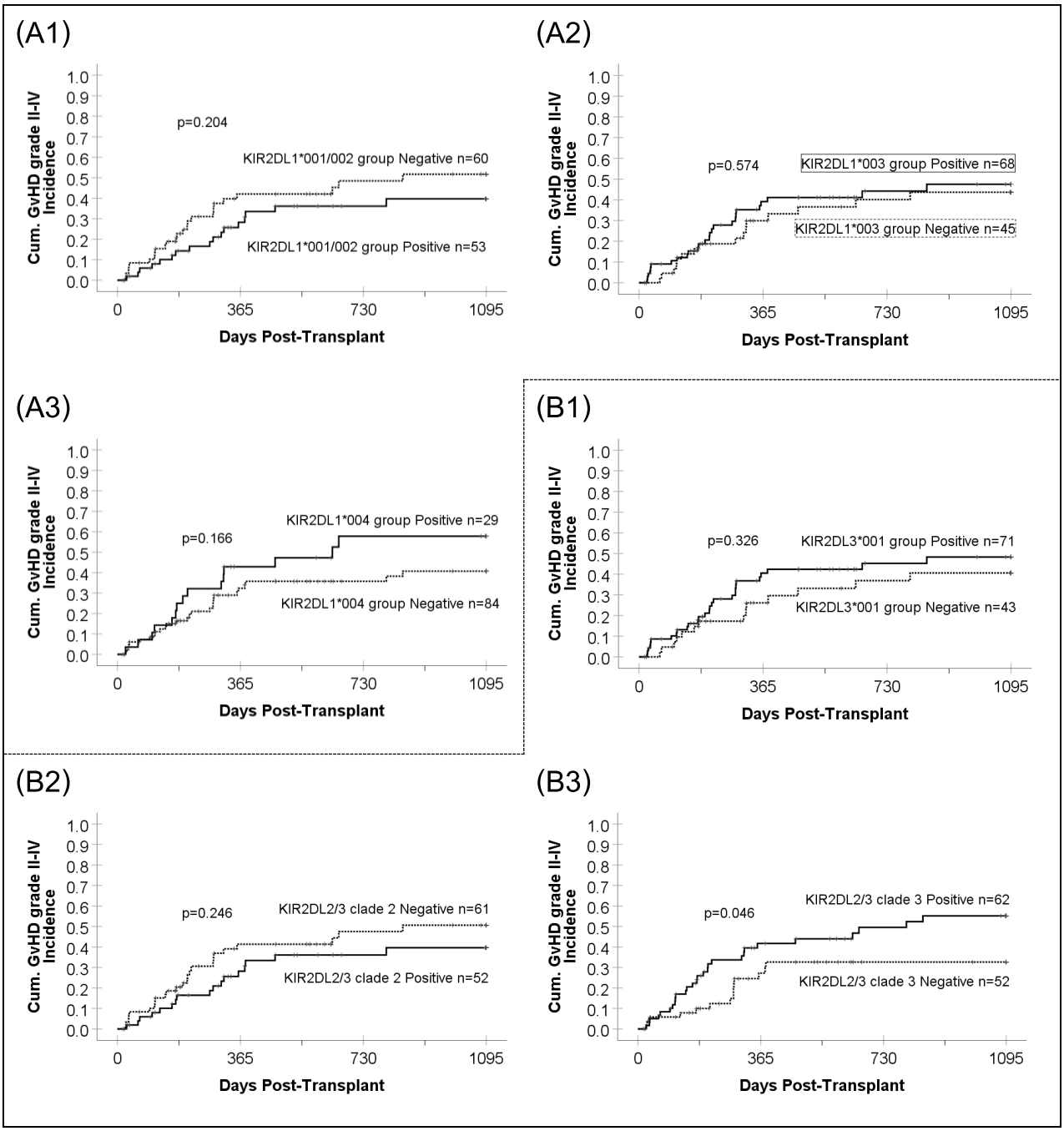


Figure 47 – The impact of donor KIR2DL1 (n=113) and KIR2DL2/3 (n=114) allele group presence/absence upon 3-year post-transplant GvHD grade II-IV incidence in Cohort A.

No KIR2DL1/3 allele groups investigated influenced 3-year NRM incidence. (A1) KIR2DL1*001 group donor presence/absence (p=0.204). (A2) KIR2DL1*003 group donor presence/absence (p=0.574). (A2) KIR2DL1*004 group donor presence/absence (p=0.166). (B1) KIR2DL3*001 group donor presence/absence (p=0.326). (B2) KIR2DL3*002 group donor presence/absence (p=0.246). (B3) Transplants using KIR2DL2*001 group positive donor significantly increased 3-year incidence of GvHD II-IV (p=0.046, p_c=0.276).

KIR Gene Group in Transplant Donor	Cohort	Category	n (%)	Overall Mortality HR (95% CI)	Non-Relapse Mortality HR (95% CI)	Events HR (95% CI)	Relapse HR (95% CI)	GvHD grade II-IV HR (95% CI)
KIR2DL3* 001 group	Cohort A (All Transplants)	Negative	43 (37.7%)	1	1	1	1	1
		Positive	71 (62.2%)	1.40 (0.80-2.45) (p=0.238)	0.64 (0.25-1.62) (p=0.347)	1.47 (0.87-2.49) (p=0.155)	1.77 (0.86-3.67) (p=0.122)	1.38 (0.73-2.62) (p=0.329)
	Cohort B (T cell Depleted RIC Transplants)	Negative	32 (36.8%)	*1*	1	**1**	**1**	1
		Positive	55 (63.2%)	*1.90 (0.98-3.67) (p=0.057)*	0.74 (0.25-2.21) (p=0.590)	**2.09 (1.11-3.94) (p=0.022)**	**2.88 (1.18-7.04) (p=0.020)**	1.18 (0.57-2.45) (p=0.657)
KIR2DL3* 002 group	Cohort A	Negative	61 (53.5%)	*1*	1	1	1	1
		Positive	53 (46.5%)	*0.61 (0.35-1.04) (p=0.068)*	1.05 (0.41-2.64) (p=0.926)	0.71 (0.43-1.17) (p=0.182)	0.73 (0.38-1.40) (p=0.343)	0.70 (0.38-1.29) (p=0.249)
	Cohort B	Negative	48 (55.2%)	**1**	1	**1**	1	1
		Positive	39 (44.8%)	**0.43 (0.23-0.81) (p=0.009)**	0.65 (0.21-1.98) (p=0.447)	**0.49 (0.27-0.88) (p=0.016)**	0.56 (0.27-1.17) (p=0.121)	0.75 (0.37-1.51) (p=0.745)

Table 38 – Comparison of hazard ratios (HR) of donor KIR2DL3*001 and 002 allele groups in transplantation Cohorts A (n=114) and B (n=87).

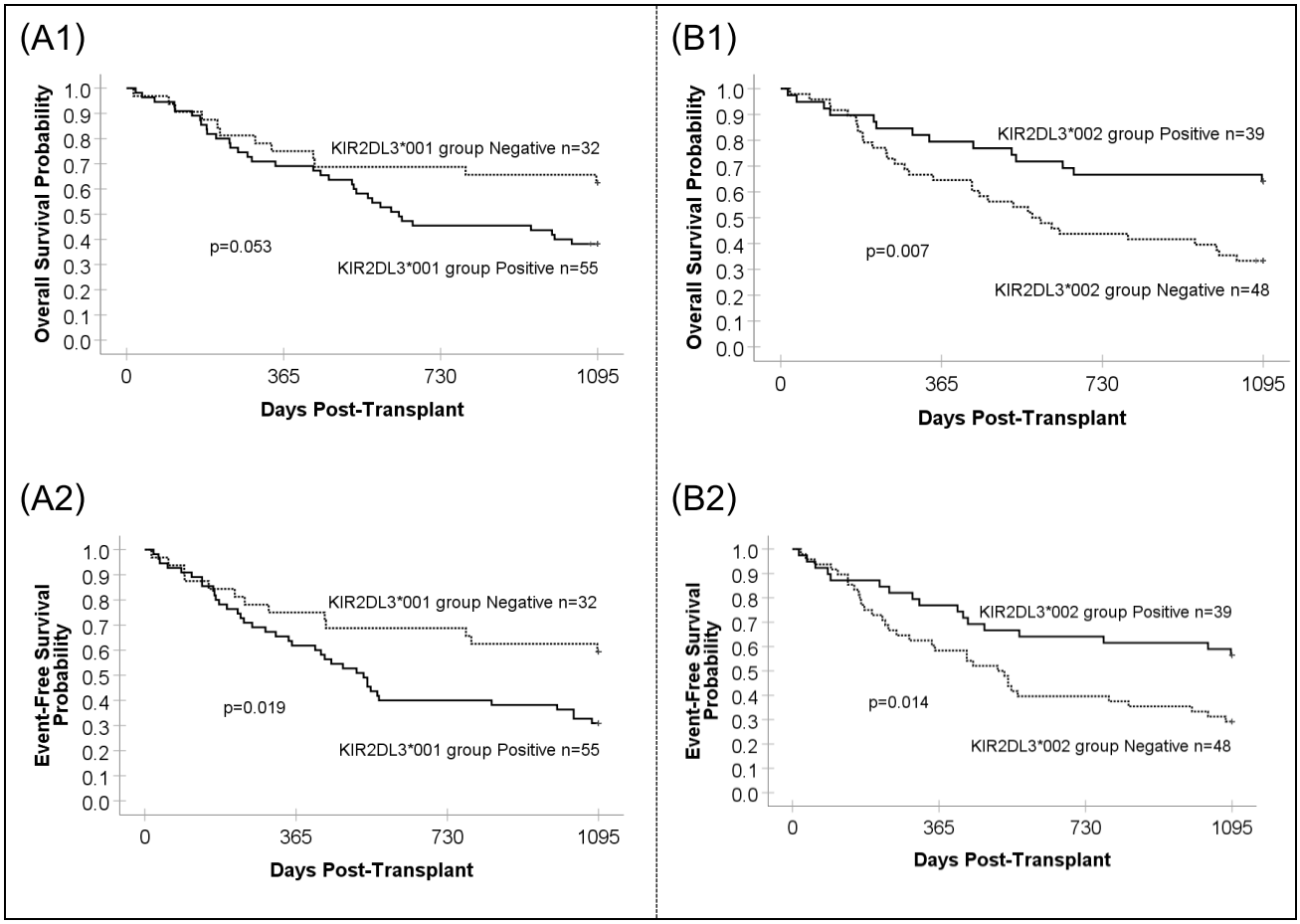


Figure 48 – Impact of KIR2DL3*001 and 002 group presence/absence in the donor upon 3-year post-transplant OS and EFS in Cohort B (n=87).

KIR2DL3*001 positive donors reduced (A1) 3-year OS (p=0.053) and (A2) EFS (p=0.019). KIR2DL3*002 improved (B1) 3-year OS (p=0.007) and (B2) EFS (p=0.014).

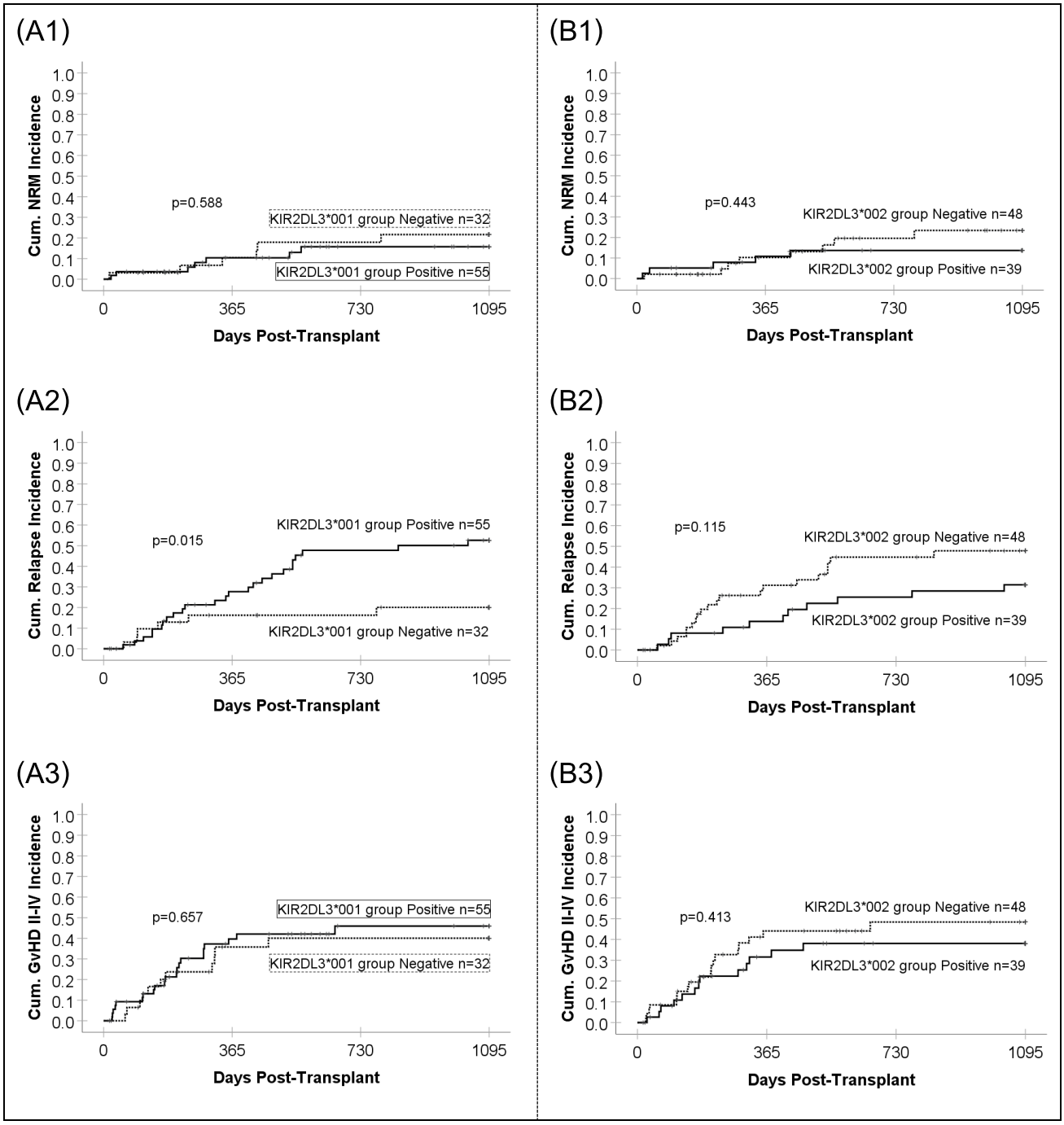


Figure 49 – Impact of KIR2DL3*001 and 002 group presence/absence in the donor upon 3-year post-transplant NRM, relapse and GvHD grade II-IV incidence in Cohort B (n=87).

(A1) KIR2DL3*001 group presence/absence in the donor did not influence 3-year NRM incidence (p=0.588). (A2) KIR2DL3*001 group positive donors increased incidence of relapse (p=0.015). (A3) KIR2DL3*001 group presence/absence in the donor did not influence 3-year GvHD grade II-IV incidence (p=0.657). (B1) KIR2DL3*002 group presence/absence in the donor did not influence 3-year NRM incidence (p=0.443). (B2) KIR2DL3*002 group positive donors displayed a weak trend towards reducing 3-year NRM incidence (p=0.115). (B3) KIR2DL3*002 group presence/absence in the donor did not influence 3-year NRM incidence (p=0.413).

Appendix A References

Cooley, S., Trachtenberg, E., Bergemann, T. L., Saeteurn, K., Klein, J., Le, C. T., Marsh, S. G., Guethlein, L. A., Parham, P., Miller, J. S. & Weisdorf, D. J. (2009) Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood,* 113(3)**,** 726-32.

Cooley, S., Weisdorf, D. J., Guethlein, L. A., Klein, J. P., Wang, T., Le, C. T., Marsh, S. G., Geraghty, D., Spellman, S., Haagenson, M. D., Ladner, M., Trachtenberg, E., Parham, P. & Miller, J. S. (2010) Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood,* 116(14)**,** 2411-9.

Leung, W., Iyengar, R., Turner, V., Lang, P., Bader, P., Conn, P., Niethammer, D. & Handgretinger, R. (2004) Determinants of antileukemia effects of allogeneic NK cells. *The Journal of Immunology,* 172(1)**,** 644-50.

Appendix B – Thesis Project Proposal

Appendix B.i – Project Proposal & Royal College of Pathologists Approval

DClinSci C2 Module Proforma

Completed forms should be emailed to admin@mahse.co.uk

Trainee Details	
Name:	Paul Andrew Wright
Student ID:	5801227
University:	University of Manchester
HSST Specialism:	Histocompatibility & Immunogenetics
Place of work:	Transplantation Laboratory, Manchester Royal Infirmary
Date proforma submitted:	30/01/2017

Research Project Details	
Research dissertation working title:	Killer Immunoglobin-like Receptors (KIR) and Haematopoietic Progenitor Cell Transplantation (HPCT): Developing a novel model for donor selection
Name of proposed workplace supervisor:	Stephen Sheldon
Contact email of proposed workplace supervisor:	Stephen.Sheldon@cmft.nhs.uk
Description of proposed research (500 words maximum) ***Please include:***	
(a) Aims of the research **1) Implement next generation sequencing of KIR genes to contribute to the clinical services provided by the Transplantation Laboratory. 2) To investigate the impact of KIR polymorphism in HPCT. Existing assessments used within the field only consider the presence of absence of activating haplotypes. 3) Evaluate existing KIR assessments and devise recommendations for a novel algorithm for KIR assessment within HPCT.**	
(b) Principal research question(s) **Does KIR polymorphism significantly impact upon HPCT outcomes? Does high resolution KIR typing provide a more sensitive predictor of HPCT outcome? How does the relationship between inhibitory and activating KIR relate to**	

overall NK cell functional capacity in the setting of HPCT?

(c) Proposed methods

For investigation into effect of KIR on transplant outcome: High resolution Next Generation Sequencing of recipient HLA and donor KIR. I will then retrospectively compare this data to transplant outcomes to calculate the correlation between KIR/HLA interactions and transplant outcome. For investigation of biological function of KIR: Surface Plasmon Resonance for measuring affinity of KIR, using the gene products from a range of KIR alleles to assess the impact of KIR polymorphism in the functional capacity of KIR. Fluorescence correlation spectroscopy is hoped to be used to map surface density of KIR on NK cells to assess variation in surface expression of KIR. I currently do not have access to the equipment necessary for Surface Plasmon Resonance or Fluorescence Correlation Spectroscopy, and so I plan to reach out to other research groups for access to this equipment and/or collaboration.

(d) Potential impact of research

Existing KIR assessments are used to select the optimal donor among a number of potential haploidentical donors (e.g. parents or siblings, who share a single haplotype with the patient). KIR compatibility is then used to choose the preferential donor among those of similar HLA match, as HLA compatibility is accepted as the principle genetic influence of transplant outcomes. By assessing the polymorphism of KIR and genotyping to the highest possible resolution, it is hoped that a more detailed understanding of KIR's influence on transplant outcome may be developed, influencing the construction of a more nuanced KIR compatibility algorithm that can further aid clinicians in selecting the optimal donor in other situations.

(e) A summary of patient and public involvement in the research

Stored patient samples will be used for investigating the influence of KIR on transplant outcomes. Cell samples will be collected from healthy volunteers for investigation into the biochemical characteristics of KIR.

Research Governance	Yes:	No:
(a) Does your proposal involve animal experimentation? If yes, do you and/or your proposed supervisor hold a valid and current animal licence? (please give details) **Click here to enter text.**	☐	☒
(b) Does your proposal involve human participants?	☒	☐
(c) Does your proposal involve samples covered under the Human Tissue Act (HTA)?	☐	☒
If you answered yes to either (b) or (c) above; • Is ethical approval required? • If required, has ethical approval been obtained? (please give details) Click here to enter text.	☒ ☐	☐ ☒

Research Costings	Yes:	No:
(a) Has the project been costed?	☐	☒
(b) Are funds in place to cover the costs? If funds are not in place, outline the approach to securing these costs: **Click here to enter text.**	☒	☐

For Office Use Only:

Approval of C2 Research Project by University	
HSST Lead:	Select HSST Lead from list.
Notes:	Click here to enter text.
Signed:	
Date approved:	Click here to enter text.

Approval of C2 Research Project by Royal College of Pathologists (Life Sciences only)	
Name:	Dr Ann-Margaret Little
Notes:	The proposed research study is at a suitable level for the Part 2 H&I written work and the findings will be of direct interest to the field of H&I. I wonder if the candidate has considered the numbers of patient/donor pairs that will need to be tested in order for significant data to be generated bearing in mind the heterogeneity within the transplanted patient cohort. The investigation of KIR expression is very important and it will be interesting to find out how this may vary within a healthy and also transplanted population – its not clear exactly what population will be studied for this part of the research.
Signed:	
Date approved:	29th March 2017

Appendix B.ii – Study Protocol

Study Title:

KIR allelic polymorphism and Haematopoietic Progenitor Cell Transplantation: Investigation into the influence of KIR on natural killer cell phenotype and transplant outcomes in AML and MPS-1H

Chief Investigator: Mr. Paul A. Wright[1]

Co-Investigators Mr. Stephen Sheldon[1]

Dr. Lynne Hampson[2]

Dr. Kay Poulton[1]

Institutions:

1. **Transplantation Laboratory**
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   Oxford Rd
   Manchester
   M13 9WL

2. **Viral Oncology, Room L5 CT 380**
   Floor 5 Research
   St Mary's Hospital
   Oxford Road
   Manchester
   M13 9WL

Study Sponsor: Manchester University NHS Foundation Trust

Sponsor Contact:
Lynne Webster

Research Office
1st Floor, Nowgen Building
29 Grafton Street
Manchester
M13 9WU

Contact **Mr Paul A Wright** 0161-276 6397

Background

The Killer Immunoglobulin Receptor (KIR) genes are used as one criterion for selecting the most suitable donor in haematopoietic progenitor cell transplantation (HPCT). These genes act as a blue print for KIR proteins, which are receptors found on the surface of Natural Killer (NK) cells. NK cells are immune cells vital for the removal of cells infected with viruses and of mutated cells which may develop into cancer.

The KIR genes vary between individuals in several ways:

1. The combination of genes present – This is known as the 'gene content' (fig 1). Two individuals may possess different sets of KIR genes, though some KIR genes are shared by the whole population.
2. Each KIR gene exists in different forms, known as alleles – This variation is known as 'allelic polymorphism' (fig. 2). Even if two individuals possess the same KIR genes, they may possess slightly different forms (alleles) of the same gene.

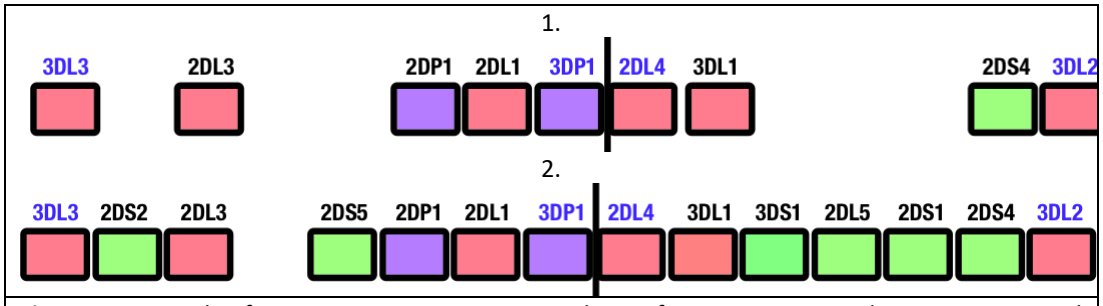

Figure 1 – Example of variation in gene content. Each set of genes represents the genes possessed by a single individual (individuals labelled 1 and 2). Some KIR genes are possessed by both individuals, but the bottom example possesses many genes the top example lacks. Individual 1 represents one of the expected combinations for Group A, while individual 2 represents one of the expected combinations for Group B.

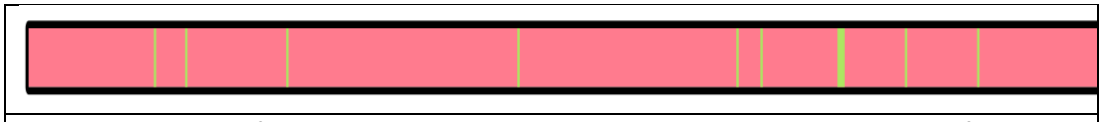

Figure 2 – Example of variation due to allelic polymorphism, comparing two alleles of the same gene: KIL3DL1*0010101 and KIR3DL1*0040101. The red represents parts of the gene sequence where the two alleles are identical. The green represents where the two gene sequences diverge. Although largely similar, these small differences influence the activity of the expressed protein.

Both of these systems of genetic variation impact upon the activity of the encoded KIR protein. The current model used predominantly in the UK considers gene content by categorising the combination of KIR genes found in an individual into one of two groups: group A or B. This model does not consider the allelic polymorphism of genes (i.e. which form of a gene is present), and so this provides an area where research is required to assess the clinical influence of this variation. Our study aims to assess the influence of allelic polymorphism on the activity of proteins encoded by KIR genes and on HPCT outcomes.

Protocol

The study protocol consists of five components:

1. **Clinical Phase**

The clinical study will assess the influence of KIR genes in the case of AML (paediatric and adult) and MPS-1H (paediatric). This will involve:

Adult AML HPCT (2012-2014): 89 = 178 samples (each transplant = 1 patient + 1 donor)

Paediatric AML HPCT (2012-2014): 9 = 18 samples

MPS-1H HPCT (2006-2015): 60 = 120 samples

These date ranges have been chosen for the homogeneity of treatment protocols within the patient groups, as well as the pragmatism of the costs involved with testing. KIR genes are not known risk factors for the development of AML or MPS-1H.

All transplant outcome data for each transplant will also be collected from patient medical records, up to the three year follow-up. As the study is retrospective, there is no control over the time-points of these data.

Data will include:

Donor chimerism data - Proportion of donor blood cells to patient blood cells. Ideally, 100% donor blood cells should be achieved. Collected at defined time points based upon local clinical policy: monthly for the first six months, then at 12 months, followed by annual assessments.

Any relapse events (in the case of AML) or loss of donor stem cell engraftment – Indication the transplant is failing.

Any complications - Such as graft versus host disease (GvHD), where the donor-derived immune system (produced by the transplanted stem cells) attacks patient cells.

Any need for further interventions - Such as a donor lymphocyte infusion (DLI), infusing additional donor cells to 'top up' the transplant if required.

Any re-transplants – Indication the transplant has failed sufficiently to require another transplant (either from the same donor or a different donor)

Survival data – Transplant-related death data

Each sample will be genotyped for KIR genes by two methods:

Presence/Absence – Typing all KIR genes at a low resolution to identify what KIR genes are present and absent. This will provide data on the overall KIR gene content for each individual.

High Resolution Typing – Next Generation Sequencing will be used for KIR2DL1, 3DL1-3, and 2DS4 to provide a high resolution genotype for these genes. This will provide data of allelic polymorphism at the genes sequenced.
NB: Due to the constraints of the project, it will not be possible to sequence all KIR genes at high resolution. The genes of interest have been chosen due to their known high degree of polymorphism (variation in the population).

Each sample will also have HLA class I sequenced at high resolution (HLA-A, B, C). HLA class I proteins are the target protein of KIR proteins.

Once genotyping for all samples has been completed, the genetic data will be compared against transplant outcome data to assess the relationship between the KIR gene system and transplant outcomes. This will include applying known clinical models to assess their clinical significance using our local data, as well as looking at novel correlations. This includes, but is not limited to:

Robust	Exploratory
B content Scoring model (Cooley, 2009; Cooley 2010) - A model that involves scoring the gene content.	The number of activating patient/donor KIR
The KIR Receptor-Ligand Model (Leung, 2004, park, 2015)	The number of inhibitory patient/donor KIR
The KIR Ligand-Ligand Model (Ruggeri, 1999)	The influence of allelic polymorphism in patient/donor KIR

Statistical analysis will assess the influence of the allelic variation of each KIR gene individually to investigate whether variation within a singular gene has a significant effect on transplant outcomes. We will also test existing models for donor selection by KIR data to assess clinical significance within our data set.

Both AML and MPS-1H groups will be analysed via univariate analysis to assess the influence of a range of factors on the outcomes - overall survival, disease-free survival, relapse, and acute graft versus host disease (aGvHD) after 3 years:

- HLA match
- TCE-matching status (assessing HLA-DPB1)
- Donor age
- Recipient age
- Recipient CMV
- Donor CMV
- Recipient CMV / Donor CMV combination

For the AML group, multivariate analysis will be used to exclude the influence of known major deciding factors in transplantation outcome:

- KIR allele
- HLA gene matching (No mismatch, 1 mismatch)
- Recipient/Donor cytomegalovirus (CMV) status match/mismatch
- Age of Donor (<30yrs, ≥30yrs)

For the MPS-1H group, multivariate analysis will assess:

- KIR allele
- HLA match
- TNC load

The success of the proposed multivariate analyses is largely dependent upon the degree of variation observed in the KIR genes. If polymorphism is extensive to the point of invalidating the use of multivariate analysis, a separate criterion for stratifying KIR alleles could be used, such as categorising alleles according to a single nucleotide site (chosen based on the location of the respective amino acid in the KIR protein).

2. Biomolecular Phase

To assess the influence of allelic polymorphism on the encoded protein, the biomolecular study will measure the activity of proteins encoded by several alleles of the same gene. Samples will be collected

from 60 healthy volunteers for this study, as the study does not directly relate to transplant data. The gene of interest for this study will be pragmatically selected, dependent upon the allelic polymorphism observed within the sample. The biomolecular study will assess two characteristics of KIR proteins:

Cell surface density – How many KIR proteins are present on the surface of the Natural Killer cell.

NK cells will be isolated from the individual. Flow cytometry techniques with labels specific for the KIR protein of interest will be used to detect the surface density.

Binding strength (known as binding affinity) - How strongly the KIR protein binds its respective HLA target.

KIR proteins will be isolated and purified from the sample, as well as the respective HLA target. Spectroscopy will be used to detect binding affinity of KIR for HLA.

Biomolecular techniques may be used to produce an artificial lipid bilayer for the KIR proteins to be embedded in. This simulates the environment of a cell wall.

Each of these two techniques will be used to test several alleles of the same KIR gene to assess the influence of allelic polymorphism. Ideally, six duplicates of the same allele from different individuals will be identified for each allele to assess natural variation of characters from the same allele as well as between alleles.

For the biomolecular phase, all samples will be anonymised with all links to identifying information removed.

3. Applying data of Biomolecular Study to Clinical Study

KIR gene products that are highly expressed and/or exhibit strong binding activity may significantly influence transplant outcomes. The aim of this phase is to apply the biomolecular data from phase 2 to the clinical data from phase 1, to assess whether functional variation of KIR gene products impacts upon transplant outcomes. In this way we hope to use basic science to provide a rationale for clinical modelling.

For statistical analysis, we expect to stratify KIR alleles of a single gene into low expression and high expression groups, as well as weak binding and strong binding groups. Finally, we will compare low expression/weak binding, low expression/strong binding, high expression/weak binding and high expression/strong binding.

4. Development of a Novel Model for Donor Selection in HPCT

If the data from the two clinical studies identifies novel correlations, it may be possible to construct a novel model for donor selection in HPCT. Depending on the data gathered in phases 1-3, this novel model may be an extension or modification of an existing model, or possibly a brand new form of algorithm.

To this end, if significance is found (or a trend observed) in KIR allelic variation, we will investigate whether applying the observed finding to an existing model improves the clinical significance of the model. For example, if a certain KIR allele or gene product feature has been found to significantly improve the transplant outcome, then this could be inserted into the B-content Scoring model. This model currently categorises donors into one of three groups. Further dividing donors into 4 or 5 groups could help clinicians in their decision as to which potential donor is most suitable for their patient.

5. Population Study

We will compile allele data from all samples sequenced at KIR as part of the previously described protocols to assess genetic and allelic frequencies as a single population. This will then be compared to the known literature.

Study Synopsis

Title	KIR allelic polymorphism and Haematopoietic Progenitor Cell Transplantation (HPCT): Investigation into the influence of KIR on natural killer cell phenotype and transplant outcomes in AML and MPS-1H		
Sponsor Name	Manchester University NHS Foundation Trust		
Medical condition or disease under investigation	AML MPS-1H		
Purpose of research proposed	• **1. Clinical Phase:** To establish whether the allelic polymorphism of KIR genes is of clinical significance to haematopoietic progenitor cell transplant outcomes. • **2. Biomolecular Phase:** To establish whether allelic polymorphism of KIR genes influences the phenotypic properties of the encoded proteins. • **3. Biomolecular/Clinical Phase:** To establish whether the phenotypic properties due to allelic polymorphism is of clinical significance to haematopoietic progenitor cell transplant outcomes. • **4. Model Phase** – Use data from phases 1-3 to construct a novel model for selecting HPCT donors in the context of KIR genes (if possible) • **5. Population Phase** – Assess frequencies of alleles typed in phases 1-3.		
	Clinical Study	**Biomolecular Study**	**Biomolecular/Clinical Study**
Primary outcome measure	The event free survival of the transplantation patients 3 years post-transplant	The cell surface density on natural killer cells of the proteins encoded by the genes of interest. The binding strength of the proteins encoded by the genes of interest.	The event free survival of the transplantation patients 3 years post-transplant
Secondary outcome measure (s)	The stability of donor cell engraftment in the first 3 years post-transplant. The overall survival of the transplantation patients 3 years post-transplant. The relapse rate in transplantation patients 3 years post-transplant.	N/A	The stability of donor cell engraftment in the first 3 years post-transplant. The overall survival of the transplantation patients 3 years post-transplant. The relapse rate in transplantation patients 3 years post-transplant.
Study Design	Retrospective cohort study	Descriptive study	Retrospective cohort study
Study Conclusion	Primary study end-point – 3 year post-transplant follow-up.	Once testing of all selected samples complete	Primary study end-point – 3 year post-transplant follow-up.
Sample Size	87 adult AML patient/donor pairs (186 samples) 9 paediatric AML patient/donor pairs (24 samples) 60 paediatric MPS-1H patient/donor pairs (30 samples)	To be pragmatically selected, dependent upon allelic polymorphism observed in available cohort, with a target of 6 replicates per allele.	87 adult AML patient/donor pairs (186 samples) 9 paediatric AML patient/donor pairs (24 samples) 60 paediatric MPS-1H patient/donor pairs (30 samples)

Summary of eligibility criteria	AML – All patients transplanted 2012-14 MPS-1H – All patients transplanted 2006-15.	Pragmatically selected to cover a breadth of allelic polymorphism. Duplicates of an allele will be tested to assess inter-allelic variation as well as intra-allelic variation.	AML – All patients transplanted 2012-14 MPS-1H – All patients transplanted 2006-15.

Procedures

	Clinical Study	**Biomolecular Study**
Screening & enrolment	Historic samples from all eligible patients will be tested. If insufficient sample remains, the patient will be contacted to request further sample collection. Consent will be pursued when new samples are required.	An advertisement for participants will be released to the local organisation. Consent forms will be given to participants at least a week prior to sample collection to allow time to consider consent.
Baseline	High resolution KIR genotyping at KIR2DL1, 3DL1-3, and 2DS4. Low resolution KIR genotyping at all KIR genes.	High resolution KIR genotyping at KIR2DL1, 3DL1-3, and 2DS4. Low resolution KIR genotyping at all KIR genes.
Follow up	Post-transplant outcome data collected from medical records - Relapse data (where applicable) - Donor chimerism data - Transplant-related mortality data - Transplant-related interventions data	N/A
Procedures for safety monitoring during the research project	It is not anticipated that serious adverse events will occur as a result of participation, as the research does not involve any patient care interventions. However, all events will be reported and forwarded to the sponsor and MREC as appropriate.	
Criteria for withdrawal of patients on safety grounds	The participant will be withdrawn at their request and their samples and records will be omitted from analysis.	

Appendix B.iii – IRAS Ethics Approval Documentation & Approval

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Mr Paul A Wright
Clinical Scientist
Manchester University NHS Foundation Trust
Transplantation Laboratory
Manchester Royal Infirmary
Oxford Road, Manchester
M13 9WL

Email: hra.approval@nhs.net
Research-permissions@wales.nhs.uk

17 September 2018

Dear Mr Wright

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**An investigation of the allelic polymorphism of genetic biomarkers in the context of haematopoietic progenitor cell transplantation outcome.**
IRAS project ID:	**234624**
REC reference:	**18/NW/0553**
Sponsor	**Manchester University NHS Foundation Trust**

I am pleased to confirm that HRA and Health and Care Research Wales (HCRW) Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

How should I continue to work with participating NHS organisations in England and Wales?
You should now provide a copy of this letter to all participating NHS organisations in England and Wales, as well as any documentation that has been updated as a result of the assessment.

This is a single site study sponsored by the site. The sponsor R&D office will confirm to you when the study can start following issue of HRA and HCRW Approval.

It is important that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details of the research management function for each organisation can be accessed here.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within the devolved administrations of Northern Ireland and Scotland.

IRAS project ID	234624

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report (including this letter) has been sent to the coordinating centre of each participating nation. You should work with the relevant national coordinating functions to ensure any nation specific checks are complete, and with each site so that they are able to give management permission for the study to begin.

Please see IRAS Help for information on working with NHS/HSC organisations in Northern Ireland and Scotland.

How should I work with participating non-NHS organisations?
HRA and HCRW Approval does not apply to non-NHS organisations. You should work with your non-NHS organisations to obtain local agreement in accordance with their procedures.

What are my notification responsibilities during the study?
The document "*After Ethical Review – guidance for sponsors and investigators*", issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

I am a participating NHS organisation in England or Wales. What should I do once I receive this letter?
You should work with the applicant and sponsor to complete any outstanding arrangements so you are able to confirm capacity and capability in line with the information provided in this letter.

The sponsor contact for this application is as follows:

Name: Lynne Webster
Email: lynne.webster@mft.nhs.uk

Who should I contact for further information?
Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is **234624**. Please quote this on all correspondence.

Yours sincerely

Simon Connolly
Senior Assessor

Email: hra.approval@nhs.net

IRAS project ID	234624

List of Documents

The final document set assessed and approved by HRA and HCRW Approval is listed below.

Document	*Version*	*Date*
Copies of advertisement materials for research participants [Biochemical Phase - Poster for participation]	6	23 July 2018
Covering letter on headed paper [Cover Letter]	1	24 May 2018
IRAS Application Form [IRAS_Form_09072018]		09 July 2018
Letter from funder [Letter from Funder - NHS HEE]	1	01 May 2018
Letters of invitation to participant [Invitation - Biomolecular Phase]	2	23 August 2018
Letters of invitation to participant [Invitation - Clinical Adult]	2	23 August 2018
Letters of invitation to participant [Invitation - Clinical Phase Child]	2	23 August 2018
Other [Patient Engagement]	1	18 May 2018
Other [Letter in Response to HRA/REC Feedback]	1	15 August 2018
Other [Clarification of Anonymisation]	1	23 July 2018
Other [Justification for Chief Investigator]	1	23 July 2018
Other [Validation Queries]		25 July 2018
Participant consent form [Clinical Phase - Adults v2]	4	23 August 2018
Participant consent form [Clinical Phase - Child]	5	23 August 2018
Participant consent form [Biochemical Phase]	4	23 August 2018
Participant information sheet (PIS) [Parents]	7	23 August 2018
Participant information sheet (PIS) [Clinical Phase - Under 8]	5	23 August 2018
Participant information sheet (PIS) [Clinical Phase - 8 to 12 yrs]	5	23 August 2018
Participant information sheet (PIS) [Clinical Phase - 13 to 15 years]	5	23 August 2018
Participant information sheet (PIS) [Clinical Phase - Adults]	9	23 August 2018
Participant information sheet (PIS) [Biochemical Phase]	7	23 August 2018
Referee's report or other scientific critique report [Peer Review - University of Manchester]	1	20 March 2017
Research protocol or project proposal [Protocol]	8	15 May 2018
Summary CV for Chief Investigator (CI) [Chief Investigator CV]	3	15 May 2018
Summary CV for student [Chief Investigator - Student - CV]	3	15 May 2018
Summary CV for supervisor (student research) [Clinical Supervisor - Stephen Sheldon CV]	1	19 February 2018
Summary CV for supervisor (student research) [Academic Supervisor - Lynne Hampson CV]	1	15 April 2018
Summary, synopsis or diagram (flowchart) of protocol in non technical language [Lay Summary]	3	24 July 2018
Summary, synopsis or diagram (flowchart) of protocol in non technical language [Protocol Flow Chart]	1	21 May 2018

IRAS project ID	234624

Summary of assessment

The following information provides assurance to you, the sponsor and the NHS in England and Wales that the study, as assessed for HRA and HCRW Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England and Wales to assist in assessing, arranging and confirming capacity and capability.

Assessment criteria

Section	Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	No comments
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	Sponsor is single participating NHS organisation. No requirement for statement of activities or schedule of events.
4.2	Insurance/indemnity arrangements assessed	Yes	No comments
4.3	Financial arrangements assessed	Yes	No comments
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments
5.3	Compliance with any applicable laws or regulations	Yes	No comments
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	No comments

Section	Assessment Criteria	Compliant with Standards	Comments
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

Participating NHS Organisations in England and Wales

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.
There will be a single participating NHS organisation where participants will be recruited and research activities will take place. If this study is subsequently extended to other NHS organisation(s) in England or Wales, an amendment should be submitted, with a Statement of Activities and Schedule of Events for the newly participating NHS organisation(s) in England or Wales. If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England and Wales which are not provided in IRAS, the HRA or HCRW websites, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net or HCRW at Research-permissions@wales.nhs.uk. We will work with these organisations to achieve a consistent approach to information provision.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and Wales, and the minimum expectations for education, training and experience that PIs should meet (where applicable).
A principal investigator will be in place at the participating NHS organisation. GCP training is not a generic training expectation, in line with the HRA/HCRW/MHRA statement on training expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken
Research activities will be completed by local staff with existing contractual arrangements.

IRAS project ID	234624

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England and Wales to aid study set-up.
The applicant has indicated that they intend to apply for inclusion on the NIHR CRN Portfolio.

Appendix C – Amendment to Thesis Project Protocol

Appendix C.i – Defence of Amendments to Project Proposal

The initial project proposal described in Appendix A.i was extremely broad, encompassing a range of molecular techniques to assess both the proteomics and genomics of KIR. This breadth was due to a number of factors, not least my personal hubris and inexperience in plotting the scope of a single project with finite time and resources. Having now spoken to other students completing doctoral theses (both part-time and full-time), and having experienced the limitations of working on a project part-time, I believe the final presented work is a far more realistic depiction of the viable working practices within the timeframe available.

While I attempted my best to fulfil all promises of the project, we were unable to source a number of key reagents for the proteomics portion of the project within a sensible timeframe for the programme's deadlines. This was unexpected, with the respective manufacturer informing us of a small delay, which was extended on five separate occasions before we finally cancelled the order. Each time we were only notified the day before or a couple of days after the promised dispatch date. Due to the central importance of these specific reagents to my proteomics work, we had no time to identify alternative sources, and so unfortunately, I was forced to exclude this portion of the project from the presented thesis.

With the narrowing scope of the project, this provided me with more time to analyse the genomic data. I generated a large wealth of data as part of this project – genotyping 281 samples for eight genes each (in addition to diplotype presence/absence), alongside additional data, such as post-transplant outcomes. Organising and analysing this data took a great deal of time, exacerbated by the part time nature of this study programme. I believe the time afforded in this pursuit increased confidence in the data presented, and reinforced the rigor of the findings presented.

With the funds made available by the exclusion of the proposed proteomic work, we were able to fund verification genotyping by an external laboratory (the Anschutz Medical Campus of the University of Colorado, Denver), using a second form of sequencing, Hybridisation-Based Targeted Enrichment Next Generation Sequencing. The subsequent data provided by this technique was a great asset to the presented

thesis, both in verifying the data I generated, and highlighting the limitations of the protocols I followed through the disparities identified. This challenged my perspective concerning my own data, leading to a more meaningful discussion of findings.

To close, I believe the work presented here is of sufficient quantity and quality to meet the described specifications of the National School of Healthcare Science, University of Manchester and the Royal College of Pathologists. I have been diligent in ensuring the presented piece of work fulfils the stated requirements of these honoured institutions.

Thank you for taking the time to assess my work.

Paul A. Wright

Appendix C.ii – Amended Project Proposal & RCPath Approval

Highlighted green text indicates parts of the original proposal that can no longer be fulfilled.

DClinSci C2 Module Proforma

Completed forms should be emailed to admin@mahse.co.uk

Trainee Details	
Name:	Paul Andrew Wright
Student ID:	5801227
University:	University of Manchester
HSST Specialism:	Histocompatibility & Immunogenetics
Place of work:	Transplantation Laboratory, Manchester Royal Infirmary
Date proforma submitted:	30/01/2017

Research Project Details	
Research dissertation working title:	Killer Immunoglobin-like Receptors (KIR) and Haematopoietic Progenitor Cell Transplantation (HPCT): Developing a novel model for donor selection
Name of proposed workplace supervisor:	Stephen Sheldon
Contact email of proposed workplace supervisor:	Stephen.Sheldon@cmft.nhs.uk

Description of proposed research (500 words maximum)
Please include:

(a) Aims of the research

1) Implement next generation sequencing of KIR genes to contribute to the clinical services provided by the Transplantation Laboratory. 2) To investigate the impact of KIR polymorphism in HPCT. Existing assessments used within the field only consider the presence of absence of activating haplotypes. 3) Evaluate existing KIR assessments and devise recommendations for a novel algorithm for KIR assessment within HPCT.

(b) Principal research question(s)

Does KIR polymorphism significantly impact upon HPCT outcomes? Does high resolution KIR typing provide a more sensitive predictor of HPCT outcome? How does the relationship between inhibitory and activating KIR relate to overall NK cell functional capacity in the setting of HPCT?

(c) Proposed methods

For investigation into effect of KIR on transplant outcome: High resolution Next Generation Sequencing of recipient HLA and donor KIR. I will then retrospectively compare this data to transplant outcomes to calculate the correlation between KIR/HLA interactions and transplant outcome. For investigation of biological function of KIR: Surface Plasmon Resonance for measuring affinity of KIR, using the gene

products from a range of KIR alleles to assess the impact of KIR polymorphism in the functional capacity of KIR. Fluorescence correlation spectroscopy is hoped to be used to map surface density of KIR on NK cells to assess variation in surface expression of KIR. I currently do not have access to the equipment necessary for Surface Plasmon Resonance or Fluorescence Correlation Spectroscopy, and so I plan to reach out to other research groups for access to this equipment and/or collaboration.

(d) *Potential impact of research*

Existing KIR assessments are used to select the optimal donor among a number of potential haploidentical donors (e.g. parents or siblings, who share a single haplotype with the patient). KIR compatibility is then used to choose the preferential donor among those of similar HLA match, as HLA compatibility is accepted as the principle genetic influence of transplant outcomes. By assessing the polymorphism of KIR and genotyping to the highest possible resolution, it is hoped that a more detailed understanding of KIR's influence on transplant outcome may be developed, influencing the construction of a more nuanced KIR compatibility algorithm that can further aid clinicians in selecting the optimal donor in other situations.

(e) *A summary of patient and public involvement in the research*

Stored patient samples will be used for investigating the influence of KIR on transplant outcomes. Cell samples will be collected from healthy volunteers for investigation into the biochemical characteristics of KIR.

Research Governance	Yes:	No:
(a) Does your proposal involve animal experimentation? If yes, do you and/or your proposed supervisor hold a valid and current animal licence? (please give details) Click here to enter text.	☐	☒
(b) Does your proposal involve human participants?	☒	☐
(c) Does your proposal involve samples covered under the Human Tissue Act (HTA)?	☐	☒
If you answered yes to either (b) or (c) above; • Is ethical approval required? • If required, has ethical approval been obtained? (please give details) Click here to enter text.	☒ ☐	☐ ☒

Research Costings	Yes:	No:
(a) Has the project been costed?	☐	☒
(b) Are funds in place to cover the costs?		

Research Costings		
If funds are not in place, outline the approach to securing these costs: Click here to enter text.	Yes: ☒	No: ☐

For Office Use Only:

Approval of C2 Research Project by University	
HSST Lead:	Select HSST Lead from list.
Notes:	Click here to enter text.
Signed:	
Date approved:	Click here to enter text.

Approval of C2 Research Project by Royal College of Pathologists (Life Sciences only)	
Name:	Click here to enter text.
Notes:	Click here to enter text.
Signed:	
Date approved:	Click here to enter text.

Paul Andrew Wright – DClinSci C2 Module –Review by The Royal College of Pathologists

Approval of Research Project by Royal College of Pathologists (Life Sciences only)		
Name of reviewer:	Dr Tracey Rees	
Select one and provide comments below:	Approved	
	Approved with recommendations for minor amendments*	√
	Action required before Approval*	
	Not approved*	
***Comments required** Note that comments provided will be shared with trainees and their supervisors	The candidate should provide information on the additional data that will be analysed to support the investigation of the impact of KIR gene polymorphism on the outcome of Haematopoietic Stem Cell Transplantation and the subsequent algorithm for donor selection.	
Signed:	Tracey Rees	
Date:	31/10/2019	

Appendix C.iii – Amended Protocol

Study Protocol Overview

Study Title:

KIR allelic polymorphism and Haematopoietic Progenitor Cell Transplantation: Investigation into the influence of KIR on natural killer cell phenotype and transplant outcomes in AML and MPS-1H

Chief Investigator: Mr. Paul A. Wright[1]

Co-Investigators **Mr. Stephen Sheldon[1]**

Dr. Lynne Hampson[2]

Dr. Kay Poulton[1]

Institutions:

3. **Transplantation Laboratory**
   2nd Floor, Purple Zone
   Manchester University NHS Foundation Trust
   Oxford Rd
   Manchester
   M13 9WL

4. **Viral Oncology, Room L5 CT 380**
   Floor 5 Research
   St Mary's Hospital
   Oxford Road
   Manchester
   M13 9WL

Study Sponsor: Manchester University NHS Foundation Trust

Sponsor Contact:
Lynne Webster

Research Office
1st Floor, Nowgen Building
29 Grafton Street
Manchester
M13 9WU
Contact **Mr Paul A Wright** 0161-276 6397

Background

The Killer Immunoglobulin Receptor (KIR) genes are used as one criterion for selecting the most suitable donor in haematopoietic progenitor cell transplantation (HPCT). These genes act as a blue print for KIR proteins, which are receptors found on the surface of Natural Killer (NK) cells. NK cells are immune cells vital for the removal of cells infected with viruses and of mutated cells which may develop into cancer.

The KIR genes vary between individuals in several ways:

3. The combination of genes present – This is known as the 'gene content' (fig 1). Two individuals may possess different sets of KIR genes, though some KIR genes are shared by the whole population.
4. Each KIR gene exists in different forms, known as alleles – This variation is known as 'allelic polymorphism' (fig. 2). Even if two individuals possess the same KIR genes, they may possess slightly different forms (alleles) of the same gene.

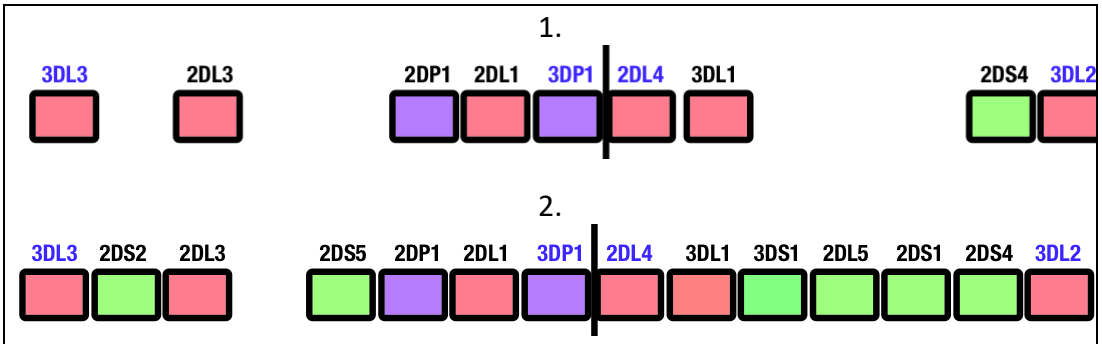

Figure 1 – Example of variation in gene content. Each set of genes represents the genes possessed by a single individual (individuals labelled 1 and 2). Some KIR genes are possessed by both individuals, but the bottom example possesses many genes the top example lacks. Individual 1 represents one of the expected combinations for Group A, while individual 2 represents one of the expected combinations for Group B.

Figure 2 – Example of variation due to allelic polymorphism, comparing two alleles of the same gene: KIL3DL1*0010101 and KIR3DL1*0040101. The red represents parts of the gene sequence where the two alleles are identical. The green represents where the two gene sequences diverge. Although largely similar, these small differences influence the activity of the expressed protein.

Both of these systems of genetic variation impact upon the activity of the encoded KIR protein. The current model used predominantly in the UK considers gene content by categorising the combination of KIR genes found in an individual into one of two groups: group A or B. This model does not consider the allelic polymorphism of genes (i.e. which form of a gene is present), and so this provides an area where research is required to assess the clinical influence of this variation. Our study aims to assess the influence of allelic polymorphism on the activity of proteins encoded by KIR genes and on HPCT outcomes.

Protocol

The study protocol consists of five components:

6. Clinical Phase

The clinical study will assess the influence of KIR genes in the case of AML (paediatric and adult) and MPS-1H (paediatric). This will involve:

Adult AML HPCT (2012-2014): 89 = 178 samples (each transplant = 1 patient + 1 donor)

Paediatric AML HPCT (2012-2014): 9 = 18 samples

MPS-1H HPCT (2006-2015): 60 = 120 samples

These date ranges have been chosen for the homogeneity of treatment protocols within the patient groups, as well as the pragmatism of the costs involved with testing. KIR genes are not known risk factors for the development of AML or MPS-1H.

All transplant outcome data for each transplant will also be collected from patient medical records, up to the three year follow-up. As the study is retrospective, there is no control over the time-points of these data.

Data will include:

Donor chimerism data - Proportion of donor blood cells to patient blood cells. Ideally, 100% donor blood cells should be achieved. Collected at defined time points based upon local clinical policy: monthly for the first six months, then at 12 months, followed by annual assessments.

Any relapse events (in the case of AML) or loss of donor stem cell engraftment – Indication the transplant is failing.

Any complications - Such as graft versus host disease (GvHD), where the donor-derived immune system (produced by the transplanted stem cells) attacks patient cells.

Any need for further interventions - Such as a donor lymphocyte infusion (DLI), infusing additional donor cells to ‘top up’ the transplant if required.

Any re-transplants – Indication the transplant has failed sufficiently to require another transplant (either from the same donor or a different donor)

Survival data – Transplant-related death data

Each sample will be genotyped for KIR genes by two methods:

Presence/Absence – Typing all KIR genes at a low resolution to identify what KIR genes are present and absent. This will provide data on the overall KIR gene content for each individual.

High Resolution Typing – Next Generation Sequencing will be used for KIR2DL1, 3DL1-3, and 2DS4 to provide a high resolution genotype for these genes. This will provide data of allelic polymorphism at the genes sequenced. NB: Due to the constraints of the project, it will not be possible to sequence all KIR genes at high resolution. The genes of interest have been chosen due to their known high degree of polymorphism (variation in the population).

Each sample will also have HLA class I sequenced at high resolution (HLA-A, B, C). HLA class I proteins are the target protein of KIR proteins.

Once genotyping for all samples has been completed, the genetic data will be compared against transplant outcome data to assess the relationship between the KIR gene system and transplant outcomes. This will include applying known clinical models to assess their clinical significance using our local data, as well as looking at novel correlations. This includes, but is not limited to:

Robust	**Exploratory**
B content Scoring model (Cooley, 2009; Cooley 2010) - A model that involves scoring the gene content.	The number of activating patient/donor KIR
The KIR Receptor-Ligand Model (Leung, 2004, park, 2015)	The number of inhibitory patient/donor KIR
The KIR Ligand-Ligand Model (Ruggeri, 1999)	The influence of allelic polymorphism in patient/donor KIR

Statistical analysis will assess the influence of the allelic variation of each KIR gene individually to investigate whether variation within a singular gene has a significant

effect on transplant outcomes. We will also test existing models for donor selection by KIR data to assess clinical significance within our data set.

Both AML and MPS-1H groups will be analysed via univariate analysis to assess the influence of a range of factors on the outcomes - overall survival, disease-free survival, relapse, and acute graft versus host disease (aGvHD) after 3 years:

- HLA match
- TCE-matching status (assessing HLA-DPB1)
- Donor age
- Recipient age
- Recipient CMV
- Donor CMV
- Recipient CMV / Donor CMV combination

For the AML group, multivariate analysis will be used to exclude the influence of known major deciding factors in transplantation outcome:

- KIR allele
- HLA gene matching (No mismatch, 1 mismatch)
- Recipient/Donor cytomegalovirus (CMV) status match/mismatch
- Age of Donor (<30yrs, ≥30yrs)

For the MPS-1H group, multivariate analysis will assess:

- KIR allele
- HLA match
- TNC load

The success of the proposed multivariate analyses is largely dependent upon the degree of variation observed in the KIR genes. If polymorphism is extensive to the point of invalidating the use of multivariate analysis, a separate criterion for stratifying KIR alleles could be used, such as categorising alleles according to a single nucleotide site (chosen based on the location of the respective amino acid in the KIR protein).

7. Biomolecular Phase

To assess the influence of allelic polymorphism on the encoded protein, the biomolecular study will measure the activity of proteins encoded by several alleles of the same gene. Samples will be collected from 60 healthy volunteers for this study, as the study does not directly relate to transplant data. The gene of interest for this study will be pragmatically selected, dependent upon the allelic polymorphism observed within the sample. The biomolecular study will assess two characteristics of KIR proteins:

> **Cell surface density** – How many KIR proteins are present on the surface of the Natural Killer cell.

NK cells will be isolated from the individual. Flow cytometry techniques with labels specific for the KIR protein of interest will be used to detect the surface density.

Binding strength (known as binding affinity) - How strongly the KIR protein binds its respective HLA target.

KIR proteins will be isolated and purified from the sample, as well as the respective HLA target. Spectroscopy will be used to detect binding affinity of KIR for HLA.

Biomolecular techniques may be used to produce an artificial lipid bilayer for the KIR proteins to be embedded in. This simulates the environment of a cell wall.

Each of these two techniques will be used to test several alleles of the same KIR gene to assess the influence of allelic polymorphism. Ideally, six duplicates of the same allele from different individuals will be identified for each allele to assess natural variation of characters from the same allele as well as between alleles.

For the biomolecular phase, all samples will be anonymised with all links to identifying information removed.

8. Applying data of Biomolecular Study to Clinical Study

KIR gene products that are highly expressed and/or exhibit strong binding activity may significantly influence transplant outcomes. The aim of this phase is to apply the biomolecular data from phase 2 to the clinical data from phase 1, to assess whether functional variation of KIR gene products impacts upon transplant outcomes. In this way we hope to use basic science to provide a rationale for clinical modelling.

For statistical analysis, we expect to stratify KIR alleles of a single gene into low expression and high expression groups, as well as weak binding and strong binding groups. Finally, we will compare low expression/weak binding, low expression/strong binding, high expression/weak binding and high expression/strong binding.

9. Development of a Novel Model for Donor Selection in HPCT

If the data from the two clinical studies identifies novel correlations, it may be possible to construct a novel model for donor selection in HPCT. Depending on the data gathered in phases 1-3, this novel model may be an extension or modification of an existing model, or possibly a brand new form of algorithm.

To this end, if significance is found (or a trend observed) in KIR allelic variation, we will investigate whether applying the observed finding to an existing model improves the clinical significance of the model. For example, if a certain KIR allele or gene product feature has been found to significantly improve the transplant outcome, then this could be inserted into the B-content Scoring model. This model currently categorises donors into one of three groups. Further dividing donors into 4 or 5 groups could help clinicians in their decision as to which potential donor is most suitable for their patient.

10. Population Study

We will compile allele data from all samples sequenced at KIR as part of the previously described protocols to assess genetic and allelic frequencies as a single population. This will then be compared to the known literature.

Study Synopsis

Title	KIR allelic polymorphism and Haematopoietic Progenitor Cell Transplantation (HPCT): Investigation into the influence of KIR on natural killer cell phenotype and transplant outcomes in AML and MPS-1H
Sponsor Name	Manchester University NHS Foundation Trust
Medical condition or disease under investigation	AML MPS-1H
Purpose of research proposed	• **1. Clinical Phase:** To establish whether the allelic polymorphism of KIR genes is of clinical significance to haematopoietic progenitor cell transplant outcomes. • **2. Biomolecular Phase:** To establish whether allelic polymorphism of KIR genes influences the phenotypic properties of the encoded proteins. • **3. Biomolecular/Clinical Phase:** To establish whether the phenotypic properties due to allelic polymorphism is of clinical significance to haematopoietic progenitor cell transplant outcomes. • **4. Model Phase** – Use data from phases 1-3 to construct a novel model for selecting HPCT donors in the context of KIR genes (if possible) • **5. Population Phase** – Assess frequencies of alleles typed in phases 1-3.

	Clinical Study	Biomolecular Study	Biomolecular/Clinical Study
Primary outcome measure	The event free survival of the transplantation patients 3 years post-transplant	The cell surface density on natural killer cells of the proteins encoded by the genes of interest. The binding strength of the proteins encoded by the genes of interest.	The event free survival of the transplantation patients 3 years post-transplant
Secondary outcome measure (s)	The stability of donor cell engraftment in the first 3 years post-transplant. The overall survival of the transplantation patients 3 years post-transplant. The relapse rate in transplantation patients 3 years post-transplant.	N/A	The stability of donor cell engraftment in the first 3 years post-transplant. The overall survival of the transplantation patients 3 years post-transplant. The relapse rate in transplantation patients 3 years post-transplant.
Study Design	Retrospective cohort study	Descriptive study	Retrospective cohort study
Study Conclusion	Primary study end-point – 3 year post-transplant follow-up.	Once testing of all selected samples complete	Primary study end-point – 3 year post-transplant follow-up.
Sample Size	**125 adult AML patient/donor pairs (186 samples)** **19 paediatric AML patient/donor pairs (24 samples)** 60 paediatric MPS-1H patient/donor pairs (30 samples)	To be pragmatically selected, dependent upon allelic polymorphism observed in available cohort, with a target of 6 replicates per allele.	**125 adult AML patient/donor pairs (186 samples)** **19 paediatric AML patient/donor pairs (24 samples)** 60 paediatric MPS-1H patient/donor pairs (30 samples)
Summary of eligibility criteria	AML – **All patients transplanted October 2011 – March 2016** MPS-1H – All patients transplanted 2006-15.	Pragmatically selected to cover a breadth of allelic polymorphism. Duplicates of an allele will be tested to assess inter-allelic variation as well as intra-allelic variation.	AML – **All patients transplanted October 2011 – March 2016** MPS-1H – All patients transplanted 2006-15.

Procedures

	Clinical Study	Biomolecular Study
Screening & enrolment	Historic samples from all eligible patients will be tested. If insufficient sample remains, the patient will be contacted to request further sample collection. Consent will be pursued when new samples are required.	An advertisement for participants will be released to the local organisation. Consent forms will be given to participants at least a week prior to sample collection to allow time to consider consent.
Baseline	High resolution KIR genotyping at KIR2DL1, 3DL1-3, and 2DS4. Low resolution KIR genotyping at all KIR genes.	High resolution KIR genotyping at KIR2DL1, 3DL1-3, and 2DS4. Low resolution KIR genotyping at all KIR genes.
Follow up	Post-transplant outcome data collected from medical records - Relapse data (where applicable) - Donor chimerism data - Transplant-related mortality data - Transplant-related interventions data	N/A
Procedures for safety monitoring during the research project	It is not anticipated that serious adverse events will occur as a result of participation, as the research does not involve any patient care interventions. However, all events will be reported and forwarded to the sponsor and MREC as appropriate.	
Criteria for withdrawal of patients on safety grounds	The participant will be withdrawn at their request and their samples and records will be omitted from analysis.	

Appendix C.iv – Substantial Amendment to Ethics Approval

Dear Mr Wright

IRAS project ID:	234624
REC reference:	18/NW/0553
Short Study title:	The role of genes in Haematopoietic Progenitor Cell Transplantation
Date complete amendment submission received:	18 April 2019
Amendment No./ Sponsor Ref:	1
Amendment Date:	27 March 2019
Amendment Type:	**Substantial**
Outcome of HRA Assessment	**This email also constitutes HRA and HCRW Approval for the amendment, and you should not expect anything further.**

I am pleased to confirm that this amendment has been reviewed by the Research Ethics Committee and has received a Favourable Opinion. Please find attached a copy of the Favourable Opinion letter.

HRA and HCRW Approval Status

As detailed above, **this email also constitutes HRA and HCRW Approval for the amendment**. No separate notice of HRA and HCRW Approval will be issued. You should implement this amendment at NHS organisations in England and/or Wales, in line with the conditions outlined in your categorisation email.

- If this study has HRA and HCRW Approval, this amendment may be implemented at participating NHS organisations in England and/or Wales once the conditions detailed in the categorisation section above have been met
- If this study is a pre-HRA Approval study, this amendment may be implemented at participating NHS organisations in England and/or Wales that have NHS Permission, once the conditions detailed in the categorisation section above have been met. For participating NHS organisations in England and/or Wales that do not have NHS Permission, these sites should be covered by HRA and HCRW Approval before the amendment is implemented at them, please see below;
- If this study is awaiting HRA and HCRW Approval, I have passed your amendment to my colleague and you should receive separate notification that the study has received HRA and HCRW Approval, incorporating approval for this amendment.

If you require further information, please contact hra.amendments@nhs.net

18/NW/0553/AM01 Please quote this number on all correspondence

Kind regards

Nina Bakhshayesh

Health Reseach Authority 3rd Floor, Barlow House | 4 Minshull Street | HRA NRES Centre Manchester | M1 3DZ

Appendix D – Published literature review

Received: 20 June 2019 | Revised: 13 December 2019 | Accepted: 22 January 2020
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REVIEW ARTICLE

HLA Immune Response Genetics WILEY

Killer-cell immunoglobulin-like receptor assessment algorithms in haemopoietic progenitor cell transplantation: current perspectives and future opportunities

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Funding information
NHS Health Education England

Abstract

Natural killer cells preferentially target and kill malignant and virally infected cells. Both these properties present compelling clinical utility in the field of haemopoietic progenitor cell transplantation (HPCT), potentially promoting a graft vs leukaemia effect in the absence of graft vs host disease and protecting against cytomegalovirus activation. Killer Ig-like receptors (KIR) play a central role in the cytotoxic action of natural killer cells, providing opportunity for improving transplantation outcomes by prioritising potential donors with optimal characteristics. Numerous algorithms for assessing KIR gene content as part of HPCT donor selection protocols exist, but no single model has been found to be universally applicable in all transplant centres. This review summarises several of the predominant strategies in KIR assessment algorithms, discussing their basic scientific principles, clinical utility and benefits to post-transplant outcomes. Finally, the review will consider how future donor selection protocols could develop towards unifying the concepts of KIR proteomics and genetics for optimising patient care.

KEYWORDS
haematopoietic cell transplantation, HPCT, HSCT, KIR, NK cells, stem cell transplantation

1 | INTRODUCTION

The cytotoxic activity of the natural killer (NK) cell delivers an innate immune defence against infection and malignancy. In recognising the absence of HLA class I expressed on the surface of the target cell, NK cells are capable of recognising abnormal cells for clearance that would otherwise be missed by adaptive immune cells.[1] To this end, the specificities of the NK cell's CD94/NKG2A and killer immunoglobulin-like receptors (KIR) for HLA class I are central to this immunosurveillance activity. CD94/NKG2A is a C-type lectin receptor capable of binding HLA-E, a constitutively expressed non-classical HLA class I protein complex that is presented on the surface of nucleated cells as part of endogenous antigen presentation. The KIR glycoproteins provide a highly specific form of recognition that is proficient in detecting down-regulation of a single HLA class I allotype, with affinity for the C1, C2, Bw4 or A3/11 epitopes present within some HLA class I protein complexes.[2-8]

The genetic complexities of KIR gene cluster have become an area of increasing interest to the Histocompatibility and Immunogenetics (H&I) community, with KIR assessment algorithms incorporated into haemopoietic progenitor cell transplantation (HPCT) programmes to provide clinically significant improvements in post-transplant outcomes. The advent of next generation sequencing (NGS) now offers the opportunity for research and clinical laboratories to investigate the polymorphism of KIR genes in far greater detail, with the ultimate goal of developing a clinical

utility for this molecular data in donor selection algorithms. This review will consider the current understanding of KIR proteomics and genetics in the context of the current and future utility of KIR analysis in HPCT donor selection algorithms.

2 | KIR PROTEIN ACTIVITY

The "Missing Self" model helped expound the mechanism by which NK cells selectively eliminate target cells, showing a correlation between the reduction of major histocompatibility complex class I expression and an inability to inhibit the cytolytic action of NK cells in a murine model.[1] In humans, NK cells bind to an array of HLA class I epitopes, with a majority of these interactions involving position 80 within the α_1 domain of HLA-Cw.[2,7,8] Approximately half of all HLA-Cw antigens contain an asparagine residue at position 80, while the remainder contain a lysine residue, categorised as C1 and C2 epitopes respectively.[9,10] Most KIR receptors are highly specific for one of these epitopes. For example, most KIR2DL2 and 2DL3 receptors bind the C1 epitope (although some display weak affinity for the C2 epitope and HLA-B antigens), and most KIR2DL1 receptors bind the C2 epitope.[3,4] Other KIR/HLA interactions include KIR3DL1 exhibiting specificity for the HLA Bw4 epitope, and KIR3DL2 binding the HLA-A3/A11 epitope found on HLA-A3 and A11 complexes.[5,6] Despite the close functional relationship between KIR proteins and their HLA class I ligands, the two gene systems are located on separate chromosomes, 19q13.4 and 6p21.1 respectively. Although meiotic division should largely negate the strength of co-evolution between two gene systems located on disparate chromosomes, there is evidence that KIR and HLA have co-evolved to maximise interactions.[3,11-13]

The relationship between inhibitory KIR and HLA class I proteins is well characterised, but the ligands of activating KIR genes remain largely elusive. KIR3DS1 has been shown to exhibit affinity for HLA Bw4 and HLA-F (a non-classical HLA class I molecule), and KIR2DS1 binds the C2 epitope.[14-18] Evidence has indicated that KIR3DS1 binds to the *HLA-B*57:01* protein/peptide complex in a peptide-dependent manner (an HLA allotype which contains the HLA-Bw4 epitope), suggesting that in some cases the presentation of the cognate peptide within the HLA molecule's peptide binding groove is necessary to form a discontinuous epitope for KIR recognition.[16] Investigations into the specificity of KIR2DS2 have also identified specificity for a novel β_2-microglobulin-independent protein, but the identity of this ligand has not yet been ascertained.[19] The same study suggests this unidentified ligand may also act as a target for KIR2DL2 and 2DL3.

3 | KIR DIVERSITY

The KIR gene cluster on chromosome 19 is composed of up to 14 KIR genes that encode inhibitory, activating and immunomodulatory receptors, as well as two pseudogenes, KIR2DP1 and 3DP1[20,21] (Nomenclature described in Figure 1).[22-25] An additional KIR pseudogene, KIR3DX1 (originally named KIR3DL0), is located separately on chromosome 19, between the leukocyte immunoglobulin-like receptor clusters.[26] Gene content is variable within the population, with high diversity observed in both the presence/absence and copy number variation (CNV) of each gene.[27] KIR genes also exhibit allelic polymorphism, providing multiple levels of intra- and inter-population variation.

Unable to undergo somatic recombination to establish variation in specificity and functional capacity like their adaptive lymphocyte counterparts, NK cells undergo variegated expression of KIR and C-type lectin receptors to constitute diverse cellular sub-populations with different functional characteristics. This is achieved by the selective silencing and expression of individual KIR genes in a stochastic manner, ultimately affecting the NK cell's phenotype, specificity and functional capacity for cytotoxic action.[28,29] It is of note that many activating and inhibitory KIR (aKIR and iKIR, respectively) appear to compete for the same ligands. Competing signals from aKIR and iKIR proteins may provide a refined degree of variance in functional capacity between NK cell subpopulations.[29]

3.1 | Gene structure

The structure of KIR genes is relatively conserved. Each gene (excluding KIR3DP1) consists of eight (the two domain KIR genes and KIR3DL3) to nine exons (the remaining three domain KIR genes).[30] Exons 1 and 2 constitute the leader sequence, exons 3-5 form the extracellular D0, D1 and D2 domains respectively. Exon 6 forms the stem, connecting the D2 domain to the transmembrane region (exon 7), with exons 8 and 9 translating to the cytoplasmic tail. Type 1 KIR2D genes (KIR2DL1-3 and 2DS1-5) possess a pseudoexon 3, resulting in proteins with only D1 and D2 extracellular domains.[31] Type II KIR2D genes (KIR2DL4 and 2DL5) lack an exon in position 4, resulting in a protein with D0 and D2 extracellular domains.[32,33] KIR2DP1 is a type I KIR2D gene, possessing eight exons and a pseudoexon 3. KIR3DL3 lacks a stem region (exon 6), with a remnant of the stem region sequence incorporated into the transmembrane region (exon 7).[34] KIR3DP1 is the shortest of the KIR genes with only exons 1-5, with some KIR3DP1 alleles (such as *KIR3DP1*003*) presenting a deletion of exon 2.[21] KIR3DP1 possesses a sixth exon, but this is derived from an intergenic region and shares no known

KIR2DL3*0020103	cA01~tB02
Key: KIR – Acronym 2D – 2 Ig-like domains L – Long cytoplasmic tail 3 – Third 2DL protein identified * - Separator 002 – Encodes member of the second series of KIR2DL3 proteins. Differs from other KIR2DL3 alleles by non-synonymous DNA substitution 01 – Differs from other KIR2DL3*002 alleles by synonymous mutation 03 – Differs from the other KIR2DL3*00201 alleles within a non-coding region	**Key:** c/t – Centromeric/Telomeric Region A/B – A/B Haplotype characteristics Two digits – Label, denoting the specific region characteristics according to presence/absence of KIR genes and copy number variation

FIGURE 1 Examples of the nomenclature for killer immunoglobulin-like receptor (KIR) alleles[22] (left), and gene content described by Pyo et al[25] and later developed by Vierra-Green et al[23,24] (right)

FIGURE 2 Table illustrating the most commonly observed killer immunoglobulin-like receptor (KIR) diplotypes (by number of populations) from a total of 625 different KIR genotypes, as of January 2018. Data collected from studies covering 171 populations and 24 861 individuals.[20] Key: Red—inhibitory gene; Green—activating gene; Purple—pseudogene; Grey—immunomodulatory gene during placentation

Hapl. Gp	ID	KIR															Populations	Individuals
		3DL1	2DL1	2DL3	2DS4	2DL2	2DL5	3DS1	2DS1	2DS2	2DS3	2DS5	2DL4	3DL2	3DL3	2DP1	3DP1	
AA	1																	170	5,861
Bx	4																	168	1,811
Bx	2																	159	2,089
Bx	3																	149	1,018
Bx	5																	143	1,324
Bx	6																	139	804
Bx	7																	121	519
Bx	8																	115	441
Bx	9																	113	297
Bx	71																	103	398

homology with any other KIR gene, and so has been denoted as exon 6b to avoid confusion.

3.2 | Gene content

The KIR gene cluster is one of the most variable regions in size and gene content within the entire genome, owing to non-allelic homologous recombination (NAHR) events resulting in the insertion and deletion of entire KIR genes into/from haplotypes. One feature that is unique to humans is the division of KIR haplotypes into two distinct categories: group A and B haplotypes (Figure 2).[11] These groups were originally distinguished by the presence or absence of a 24 kb band when digested by *Hin*dIII restriction enzyme and analysed by Southern Blot technique.[20,27,35,36]

With some exceptions, both group A and B haplotypes contain the framework genes KIR2DL4, 3DL2, 3DL3 and pseudogene KIR3DP1 (Figures 2, 3). Group A haplotypes consist of a largely homogeneous set of up to seven genes and a second pseudogene: KIR3DL1, 2DL1, 2DL3, 2DS4 and 2DP1, accompanied by the four framework genes. The number of genes observed within group B haplotypes vary considerably, but are broadly defined by the inclusion of further aKIR and iKIR genes in assorted combinations.[20] Consequently, individuals are often categorised as possessing an A/A diplotype (for A haplotype homozygous) or B/x diplotype (for A/B haplotype heterozygous or B/B haplotype homozygous). Because of the inclusion of several aKIR genes, group B haplotypes are regarded as the "activating" haplotype.

A hotspot for recombination is positioned in the centre of the KIR gene cluster (between KIR3DP1 and 2DL4),

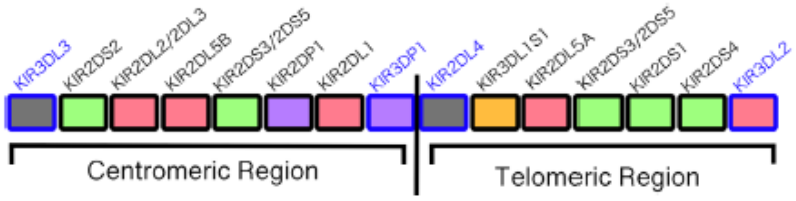
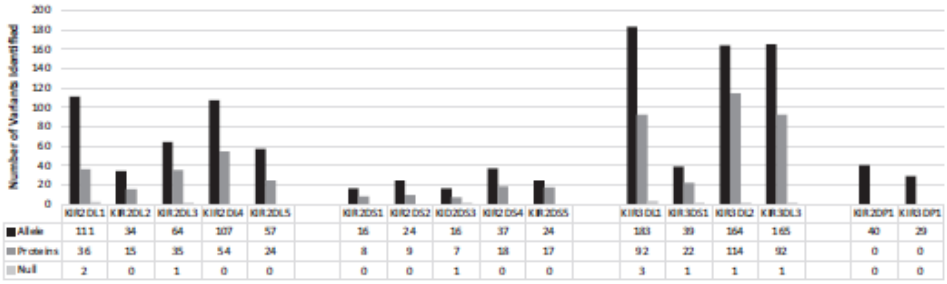


FIGURE 3 Diagram showing order and regional location within the killer immunoglobulin-like receptor (KIR) gene cluster. Each KIR haplotype will contain a different gene content comprising of the four framework genes accompanied by an assortment of non-framework genes (Table 1).[20] Hotspot for recombination indicated by vertical line. Key: Red—inhibitory gene; Green—activating gene; Purple—pseudogene; Gold—inhibitory/activating gene dependent upon allele present; Grey—immunomodulatory gene during placentation; Genes in blue border/typeface—framework genes



	KIR2DL1	KIR2DL2	KIR2DL3	KIR2DL4	KIR2DL5	KIR2DS1	KIR2DS2	KIR2DS3	KIR2DS4	KIR2DS5	KIR3DL1	KIR3DS1	KIR3DL2	KIR3DL3	KIR2DP1	KIR3DP1
Allele	111	34	64	107	57	16	24	16	37	24	183	39	164	165	40	29
Proteins	36	15	35	54	24	8	9	7	18	17	92	22	114	92	0	0
Null	2	0	1	0	0	0	0	1	0	0	3	1	1	1	0	0

FIGURE 4 Number of alleles, respective proteins, and null genes identified for each killer immunoglobulin-like receptor (KIR) gene as of the latest statistical IMGT release, December 2019.[39] NB: KIR3DL1 and KIR3DS1 have been identified as alleles of the same gene, KIR3DL1S1,[22] but have been separated in this chart to show the variation observed within each functional isoform

dividing the gene cluster into two regions: the centromeric region (region closest to the centromere of the chromosome) and telomeric region (region closest to the telomere of the chromosome) (Figure 3).[37,38] The linkage disequilibrium between genes within each region has led to the theory that most common haplotypes are derived from a limited assortment of centromeric and telomeric regions arranged in differing combinations.[24,25] As such, it is possible for a haplotype to consist of a combination of a centromeric region with the characteristics of an A haplotype and telomeric region with the characteristics of a B haplotype, or vice versa. These characteristics have inspired the development of haplotype nomenclature described in Figure 1.

3.3 | Allelic polymorphism

The presence or absence of a gene only provides partial information on KIR genetics and proteomics. KIR genes are highly polymorphic, with 977 alleles identified as of the latest IMGT statistical release in December 2019 (Figure 4).[39] This polymorphism appears to have arisen in association with the evolution of gene content variation, with population studies identifying linkage disequilibrium between several KIR gene polymorphism and A or B haplotypes.[40] The variety of defined KIR alleles is rapidly expanding, with each new study of KIR alleles likely to identify novel and previously unpublished alleles. This has led to the suggestion that the degree of polymorphism of KIR in the global population may ultimately equal or exceed that of HLA, currently accepted as the most polymorphic gene system in the human genome.[41]

Allelic polymorphisms can significantly alter the affinity and specificity of KIR proteins for their target epitope. For example, the KIR receptor encoded by *KIR2DL1*022* has specificity for the C1 epitope (in contrast to the C2 specificity observed in a majority of KIR2DL1 receptors) due to a methionine to lysine substitution at position 44.[3] Similarly, KIR2DL1 allotypes within the centromeric region of haplotype A (CenA) display higher avidity for C2 epitopes than those of haplotype B (CenB). The weaker receptor avidity of CenB KIR2DL1 allotypes are largely determined by four positions within the D2 domain: positions 114, 154, 163 and 182.[3] In

addition, some CenB KIR2DL1 allotypes are further impaired by polymorphisms within the transmembrane domain acting as a key determinant of limited surface expression.[3] Conversely, the allotypes of C1-specific iKIR within CenB haplotypes (predominantly KIR2DL2) exhibit higher avidity for both C1 epitopes than those of CenA haplotypes (KIR2DL3).[3] The C1-specific iKIR of B haplotypes also displaying a degree of cross-reactivity for C2. These findings show both the impact of genetic polymorphism upon KIR phenotypical properties and the significant disparity between the activity of KIR proteins encoded by group A and B haplotypes.

Wider phenotypical variation can be influenced by allelic polymorphism. One common allele of KIR2DS4, *KIR2DS4*0030101*, possesses a gene truncation upstream of the transmembrane domain.[42,43] The phenotypical impact of this mutation is unknown. If expressed, it has been suggested that the gene product may be secreted into the surrounding environment to quench soluble HLA, enhancing NK cell activity.[43] Allelic polymorphism can also dictate the broad signalling functionality of the KIR protein, with KIR3DL1 (an iKIR) and 3DS1 (an aKIR) found to be alleles of the same gene, KIR3DL1S1.[22] In spite of this, due to the difference in functionality, KIR3DL1 and 3DS1 continue to be considered separately in many studies.

3.4 | Copy number variation

The same NAHR events that give rise to variation in gene content in KIR haplotypes also result in CNV, presenting multiple copies of the same gene within a single haplotype.[44] As with gene content variation, NAHR is predominantly observed in group B haplotypes, with one study finding >80% of B haplotypes displaying evidence of NAHR compared to <1% in A haplotypes in a cohort of US/UK families - further evidence of the conserved nature of A haplotypes.[37] This data strengthens the theory that NAHR (and consequently, CNV) is more important to the evolution of the B haplotype than the A haplotype, but also raises questions concerning the evolutionary value of duplicating genes.

Possessing multiple copies of a KIR gene increases the likelihood that gene will be expressed, increasing the prevalence of NK cell subpopulations presenting the respective receptor.[45,46] However, CNV does not appear to increase the surface density of the KIR receptor, nor enhance the functional responses of the NK cell. These findings suggest CNV may hold clinical significance in the context of NK cell subpopulation composition within an individual, but not at the level of an individual NK cell.[45]

The NAHR that governs CNV of KIR appears to be spatially restricted, with an inconsistent degree of CNV between KIR genes. The genes at the extremities of the cluster, such as KIR3DL2, 3DL3, 2DS2, 2DS1 and 2DS4, present limited duplication in comparison with the high frequency of duplication observed in KIR3DL1S1 at the centre of the cluster.[37,47] The tight restriction of NAHR within the limits of the KIR gene complex suggests this mechanism is of central importance to the genetic evolution of the KIR gene cluster, and may explain why the primary hotspot for recombination is located at the centre of the gene cluster.

3.5 | Expression of KIR genes

The expression of KIR genes is clonally distributed in a variegated manner as a result of stochastic expression selectively silencing KIR genes via methylation, so that no single NK cell expresses all KIR genes contained within the genome.[29,48] KIR genes are hypo-methylated by default, suggesting other epigenetic mechanisms may silence KIR genes in immature NK cells.[49] Each KIR gene has two promoters: a distal unidirectional promoter and a proximal bidirectional promoter. By simultaneously transcribing from the distal sense strand and the proximal antisense strand, it is possible to produce dsRNA, which has been shown to epigenetically mediate methylation of other genes.[50] The one exception to this epigenetic mediation is KIR2DL4, which is constitutively expressed.[48,51] The promoter of KIR2DL4 does exhibit small variances in structure compared to that of clonally distributed KIR genes, although this does not appear to be sufficient to solely explain the large difference in expression characteristics.[51]

With variegated stochastic expression, no KIR phenotype represents greater than 7% of the overall NK cell population in the peripheral circulation.[29,52] The proportion of phenotypes varies between individuals, with evidence of genetic, epigenetic and environmental influences. In all cases, the largest sub-population of NK cells presents no KIR on its cell surface (41%-72% of NK cells), with expression of NKG2A providing the primary NK cell immunosurveillance activity.[29,52] Of NK cells with KIR expression, most express between one and three iKIR, with suggestions that cells with fewer iKIR have a larger presence in the peripheral circulation to facilitate rapid expansion to therapeutic levels for an effective immune response when required in times of infection.[29] NK cells with several KIR expressed provide a more specific immune defence, which requires a longer period of time to constitute the necessary therapeutic levels due to lower constitutive numbers, acting in a similar time frame to that of the adaptive immune system.[29] This observation strengthens the notion of NK cells acting as a bridge between the innate and adaptive immune systems.

4 | KIR AND HAEMOPOIETIC PROGENITOR CELL TRANSPLANTATION

4.1 | KIR analysis in donor selection

The immunoregulatory role of NK cells presents an effective clinical utility in HPCT. HLA matching remains the primary tool for selecting optimal donors, with KIR genes providing a potential supplementary selection tool for donors of equal HLA match, age and cytomegalovirus (CMV) serostatus match in accordance with existing recommendations.[53-55] With the patient and donor possessing different KIR genes and ligands, patient/donor pre-transplant assessments for selection of the optimal donor present a complex set of interactions to study in the context of HPCT outcomes and the patient's diagnosis. As KIR and HLA genes are located within different chromosomes, even siblings who are HLA matched only present a 25% probability of also being KIR matched, due to the Mendelian model of genetic inheritance. This opens the opportunity for assessing KIR even in cases of potential HLA matched sibling donors. Several competing models in assessing the impact of KIR compatibility on HPCT outcomes have been devised, but a majority share a primary aim of increasing donor NK cell reactivity towards patient cells to preferentially target malignancy (Table 1).

The wealth of data concerning the influence of KIR genes in HPCT outcomes is confounding in its diversity of findings and lack of a universally applicable model that works successfully in all transplant centres. Conflicting findings are numerous, with few commonalities in treatment, patient cohorts and study protocols between centres. The lack of standardisation across study protocols hinders the ability to optimise clinical models with the information available, and obfuscates the relevance of any direct comparison between studies. Areas of variability include the definition of KIR compatibility, the large number of variable factors associated with HPCT programmes (such as diagnosis, disease stage, other donor selection criteria, donation unit pre-treatment and conditioning regimes), and the small sample sizes involved in many studies. In spite of this, common themes are emerging in the field, showing promising signs of progress in assessing clinical outcomes. It is good practice for any transplant centre that uses KIR

TABLE 1 Summary of several models for assessing the KIR compatibility between HPCT recipient and donor

Model	Description	Most advantageous donor as described by model
KIR Receptor-Ligand Model (RLM)[65-67]	A KIR mismatch is described as the donor possessing inhibitory KIR for which the recipient lacks the ligand (ie, HLA Class I epitope).	RLM KIR mismatched donor
KIR Ligand–Ligand Model (KLM)[70]	A KIR mismatch is described as the donor possessing a KIR ligand (ie, HLA Class I epitope) which is absent in the patient.	KLM KIR mismatched donor
Missing Licencing Proof Model[73,74]	A "Missing Licencing Proof" is described as a lack of a cognate aKIR/iKIR ligand in the patient for which the donor has licenced NK cells (ie, donor possesses inhibitory KIR receptor and cognate ligand, patient lacks cognate ligand)	Donor without missing licencing proof. (recipient ligand^{+} where donor is i/aKIR^{+}/ligand^{+})
KIR B-Content Scoring Model[56,59]	Transplants from donors possessing a KIR B haplotype present improved outcomes post-transplant due to the presence of stimulatory KIR genes.	Donor possessing KIR CenB/B haplotypes
KIR Matching Model[67,77]	The KIR diplotype between patient and donor is matched in an HLA matched sibling donor setting. Compared either as a complete diplotype, or with iKIR and aKIR matching assessed separately.	KIR matched sibling (if a complete match is not possible, iKIR-matching is preferred to aKIR-matching)
Missing KIR Ligand Model in Autologous HPCT[69]	In autologous transplantation, a KIR mismatch is described as the patient possessing an inhibitory KIR for which they do not possess the ligand (ie, HLA Class I epitope).	N/A (Only applicable to autologous transplantation)

Abbreviations: NK, natural killer; HPCT, haemopoietic progenitor cell transplantation; KIR, killer immunoglobulin-like receptors.

assessments in their HPCT donor selection algorithms to assess the clinical effectiveness of their KIR assessment protocol on their own cohort of patients to ensure the respective protocol is of clinical value locally.

4.2 | Gene content models for donor selection

HPC donors possessing KIR B/x diplotypes are widely accepted to present the optimal option for HPCT when choosing between donors who are of an equal HLA match with the patient.[56-60] CenB regions in particular have been noted to provide significant protective effects in Acute Myeloid Leukaemia patients.[59-62] The beneficial effects of KIR B/B donors are greater than double those observed when using KIR A/B diplotype donors, indicating that A haplotypes may also have a causative detrimental impact upon HPCT outcomes.[59]

The KIR B Content Scoring model is a commonly used HPCT donor selection algorithm derived from these findings, prioritising B haplotype donors due to their activating characteristics (Table 2).[56,59,60] This algorithm categorises potential donors as "Neutral," "Better" or "Best." with these categories representing approximately 70%, 20% and 10% of the international donor registry pool respectively.[63] The B content ranking system places Cen-B/B donors as the optimal option, as a high B-content in the donor is likely to result in increased NK cell activation. For ease of interpretation, a "Donor KIR B-content Group Calculator" has been provided for free by the European Bioinformatics Institute, allowing transplant centres to accurately categorise their prospective donor's KIR content.[59,64]

The Receptor-Ligand Model (RLM) is a competing model that aims to mismatch donor iKIR and recipient iKIR ligand to reduce donor NK cell inhibition, and consequently increase NK cell cytotoxic activity.[65-67] This model describes a KIR mismatch as the absence of a KIR ligand in the patient that is recognised by the donor's KIR repertoire, taking advantage of the "missing self" model to drive NK cell activation. KIR mismatching using the RLM shows a trend towards improved overall survival and disease free survival, as well as decreasing the risk of relapse.[65,66] As a precautionary measure, the RLM includes a recommendation that the donor NK cells exhibit at least one inhibitory KIR specific for a recipient ligand to maintain NK cell immunoregulatory function, and minimise the risk of NK cell autoimmunity post-transplant, although no case of such autoimmunity has yet been observed.[65,68] The RLM has also been found to be clinically significant in cases of autologous HPCT, with patients possessing KIR for which they do not possess the ligand experiencing improved rates of disease-free survival following transplant.[69] As autologous transplantation by its nature does not involve donor selection, this observation is of limited clinical utility.

The KIR Ligand-Ligand Model (KLM) functions similarly to the RLM, but defines a KIR mismatch as the absence of a KIR ligand in the recipient that is present within the donor ligand repertoire (a graft vs host [GvH] mismatch).[70] As with the RLM, KIR mismatches in the KLM offer an improved overall survival rate.[71] This data is further supported by the finding that host vs graft (HvG) KIR ligand mismatches result in a higher risk of relapse (hazards ratio [HR] = 10.7, $P = .002$) and lower 2-year disease free survival (HR = 3.4, $P = .025$) compared to GvH mismatches.[72] Not all laboratories have access to KIR genotyping techniques, and so the KLM may provide value to these centres. Unfortunately, mismatching KIR ligands necessitates the mismatching of HLA genes—one of the most significant factors in post-transplant outcomes.[54] The detrimental effect of HLA mismatching must be considered when using a KIR ligand mismatching model. There are few comparative analyses to draw conclusions on comparative clinical relevance between the relatively similar KLM and RLM models, and so more research is required to ascertain which model is more beneficial to patient care.[65]

Further research has focussed on improving the clinical relevance of the RLM model influenced by considering basic scientific principles. A three level Missing Licencing Proof model has been constructed that enhances the RLM model to consider the effect of NK cell licencing on donor selection.[73,74] Both the KIR and HLA profiles of the donor are assessed, with a presumption that the presence of an KIR gene coupled with the presence of the cognate KIR ligand gene in the donor will result in the donor possessing licenced NK cells expressing that iKIR gene. Conversely, if the donor possesses the iKIR, but not its ligand, the respective donor NK cell subpopulation will not fulfil licencing. The HLA profile of the patient is then assessed for KIR ligands to ascertain whether the patient possesses the KIR ligands for the donor's licenced NK cells. This model has been applied in the context of iKIR and aKIR genes. These studies found that HPCT recipients who possessed the cognate iKIR ligands for the donor's licenced NK cells experienced superior overall survival in leukaemia patients compared, suggesting the presence of these ligands in the recipient promote immunosurveillance of malignancy post-transplant.[73] A similar beneficial effect was observed in the case of aKIR, with improved progression-free survival and reduced rate of disease progression associated with aKIR licencing proof.[74]

The findings of the iKIR missing licencing proof models appear antithetical to the principles of the RLM, where absence of the cognate ligand in the patient was

TABLE 2 Definition of the KIR B-content categories used to rank potential HPCT donors[59]

KIR B-content category	Definition
Neutral	KIR B-content score ≤ 1 (2DL3 present, 2DS2 and/or 2DL2 absent, 3DL1 and 2DS4 present)
Better	KIR B-content score ≥ 2 Cen-A/x, Tel-B/x (2DL3 present, 3DS1 and/or 2DS1 present)
Best	KIR B-content score ≥ 2 Cen-B/B, Tel-x/x (2DL3 absent, 2DS2 and/or 2DL2 present)

Note: The KIR B-content scoring system is based upon splitting each KIR haplotype into the centromeric and telomeric regions, resulting in four regions (Cen-X/X, Tel-X/X). Each region that presents B haplotype gene content is scored one, producing a final score between 0 and 4 (eg, Cen-B/B, Tel-A/A would score 2 for KIR B-content).
Abbreviations: HPCT, haemopoietic progenitor cell transplantation; KIR, killer immunoglobulin-like receptors.

found to be beneficial. Nowak et al argue that these opposing findings are due to the lack of consideration for the influence of NK cell licencing in many "missing self" models, with this additional layer of analysis providing details of the donor NK cells' ability to provide a fully licenced anti-malignancy effect.[73,74] Without fulfilling licencing of NK cells via their KIR, the lymphocyte will be largely hyporesponsive to stimulus.[75,76] The proportion of HPCT recipients with this detrimental missing licencing proof within these studies was small (<5%), and so a larger study is required to verify these results. This is likely an indication that relatively few patients may benefit from implementation of the missing licencing proof model.

In conflict with the majority of studies assessing the influence of KIR mismatching, some groups have investigated matching KIR diplotypes between donor and recipient, either as a complete diplotype or by assessing iKIR and aKIR genes separately.[67,77] KIR matching has been found to reduce risk of chronic graft vs host disease (cGvHD), with iKIR matching presenting a stronger influence in outcomes over aKIR.[67,77] No correlation were found with relapse rates, acute graft vs host disease (aGvHD) or survival, but reduced relapse and improved relapse-free survival was observed when the RLM model was applied to the KIR-matched group.[67] Unfortunately, applying HLA matching, KIR matching and the RLM model in parallel is only applicable to patients who present a KIR/Ligand mismatch within their own genotype, and greatly reduces the optimal potential pool of donors who fit these criteria when assessed in parallel with other established criteria (such as CMV serostatus and age of donor).

As the presence of ligands is essential to the licencing process of NK cells, recipient KIR ligand diversity presents further context for KIR gene content assessment models. C1/C1 recipients present improved overall survival post-HPCT and reduced risk of relapse compared to C1/C2 and recipients, but this is offset by an increased risk of grade III/IV aGvHD.[78-81] The detrimental effect of lacking the C1 epitope (approximately 15% of the European population) is well characterised, with C2/C2 recipients experiencing reduced overall survival and disease-free survival, and increased incidence of relapse compared to C1 positive recipients.[78,79] iKIR specific for C1 are the first NK cell effector receptors to be expressed during post-transplant immune reconstitution, and so early phase NK cells in C2/C2 patients fail to undergo licencing, greatly reducing the functional capacity of NK cells in the initial months post-transplant.[82,83]

These findings have raised concerns that C2/C2 patients represent an underserved demographic, who could be aided by improved donor selection protocols. It has been suggested that donor selection based on donor KIR gene content could have further beneficial and detrimental effects on the transplant outcomes of C2/C2 patients. KIR2DS1 positive donors may offer reduced relapse incidence for C2/C2 patients, but also increase the risk of transplant-related mortality (TRM).[78-80,84] KIR2DS2 positive donors may also be beneficial for C2/C2 patients receiving a 9/10 HLA matched graft, reducing TRM without affecting relapse rates.[79] Unfortunately, established donor selection algorithms, such as the B content scoring model, may offer no benefit for C2/C2 patients.[60,67]

4.3 | Limitations of assessing gene content in HPCT

The clinical models for selecting HPCT donors described thus far have presented a convincing argument for clinical consideration of KIR genes in HPCT. However, there remains a large knowledge gap between our understanding of the KIR genetics/proteomics and the structure of clinical models, making it difficult to explain precisely why and how these models work. Closing this gap requires further scrutiny and advancement in our understanding of both the basic science of KIR and the clinical outcomes of HPCT.

The gene content models described largely neglect to assess the true degree of genetic variation within the KIR gene system. The aKIR content of B haplotypes varies widely, from between two and six activating receptor genes, but these haplotypes are often treated within algorithms as a single homogenous group. In addition,

studies into NK cell activity have provided evidence for the functional variation between both different KIR genes and different alleles of the same KIR gene.[8,5,86] Assessment of the full extent of genetic variation could be of clinical benefit, further stratifying potential HPCT donors in the context of their KIR genotype. Further analysis of KIR gene content and polymorphism is required to ascertain the value of fully assessing the complexities of KIR genetics, and how these factors may be weighted for optimal clinical utility.

A common theme in the described clinical models is the objective of increasing the likelihood of activating donor NK cells. For example, the RLM aims to ensure maximal NK cell activation by minimising NK cell inhibition, while the B-content scoring model ensures maximal NK cell activation by selecting donors with superior aKIR gene content, regardless of the patient's HLA profile. It may be possible to unify these two concepts into a single model, but it is likely that a properly conducted prospective double blinded multi-centre clinical study of a large patient cohort is required to assess the true clinical significance of a more complex model.

Finally, a pragmatic strategy must be used when considering the practical utility of more complex models. Many patients do not have access to a wide pool of potential donors to select from, and so an overly complex algorithm may not be effective or efficient for donor selection. Even when considering the large pool of donors available from international bone marrow donor registries, there are then cost implications in ordering and testing samples from many prospective donors to identify the optimal donor. For example, to identify a better or best category unrelated donor, in accordance with the B Content Scoring model, it is recommended to order at least four donors (if selected from a pool of donors with no KIR genotyping available).[63] A more complex model would likely necessitate ordering further donors. This fiscal burden on clinical laboratories could be reduced by donor registries providing KIR genotyping of donors at the point of registration, but there is currently little impetus to take on this additional testing. With the growth of research into KIR genetics, there are initial indications of some registries taking on this typing, with Deutsche Knochen Mark Spenderdatei (DKMS) currently undergoing KIR genotyping of their patient population, with a projection to provide allele-level KIR genotyping data on 5 million donors by 2021.[87]

4.4 | Assessing allelic polymorphism in HPCT

Investigation into the functional heterogeneity of KIR encoded by different alleles of a single KIR gene has encouraged the development of research assessing the influence of allelic polymorphism in HPCT. At its most basic level, polymorphisms can be used to identify potentially non-functional or non-expressed genes. For example, KIR3DL1 proteins can be stratified by the presence of serine or leucine in position 86 within the D0 domain. A majority of allotypes contain serine in this position, resulting in surface expression of the KIR3DL1 protein, but eight series of KIR3DL1 proteins contain leucine, disrupting protein folding and blocking the protein's release from the endoplasmic reticulum.[10,86] One study found approximately 12.2% of HPCT donors (from a predominantly European Caucasian cohort) possessed a KIR3DL1 allotype that was retained intracellularly.[88] It has been suggested that intracellular retention of KIR may aid NK cell licencing and/or ensure minimal inhibition of NK cell activity.[89] Other studies have assessed the influence of *KIR2DS4*030101* in donors, although data is conflicting.[90,91]

Initial assessments of polymorphism have measured the influence of single KIR genes upon post-transplant outcomes, categorising transplants on the basis of selective typing of single nucleotide polymorphisms known to be influential in the phenotypical characteristics of the expressed receptor, unifying the basic scientific principles of KIR genetics and proteomics. One of the first candidate KIR genes to be considered in influencing post-transplant outcomes was KIR2DL1. Dimorphism at position 245 (within exon 7, encoding the transmembrane domain) significantly alters the signalling strength of the expressed protein: KIR receptors with arginine at this position (R^{245}) recruit more signalling complexes than receptors with cysteine at the same position (C^{245}).[92] HPCT involving donors positive for KIR2DL1-R^{245} (homozygous or heterozygous) presented superior post-transplant outcomes than recipients negative for this allotype, with improved overall survival and progression-free survival.

Outside of HPCT, several studies have analysed the relationship between allelic polymorphisms and proteomics. Allelic polymorphism can lead to differences in the prime phenotypical characteristics of a receptor: binding affinity, surface density and/or overall signalling strength.[85] Polymorphism of KIR3DL1 results in alleles with either high (such as *KIR3DL1*001*, *002*) or low (such as *KIR3DL1*005*, *007*) expression levels.[85] Polymorphism within both KIR and its ligands (HLA-Bw4 exhibits polymorphism in three key positions) also influences the binding affinity of the receptor/ligand interaction.[6,85,93] Together, these features directly influence the signalling strength of the NK cell. This has led to the suggestion that, in the context of cellular functional capacity, high surface density of KIR can compensate for weak binding affinity, but strong KIR binding affinity cannot

compensate for low surface density.[85] Furthermore, mutagenesis studies have shown HLA polymorphism outside of the Bw4 epitope can also influence binding of KIR, possibly due to changes in the tertiary structure of HLA and/or peptide binding repertoire.[94] More studies are required to assess the influence of this relationship between surface density and binding affinity upon HPCT outcomes, and to investigate the relative influence of each KIR gene within the diplotype.

4.5 | Mechanisms of action: direct or indirect activity?

The exact mechanism of action for how NK cells influence post-transplant outcomes remains unclear, but has important implications for how KIR may be assessed in HPCT donor selection. For example, the potent graft vs leukaemia (GvL) effects observed in the initial months immediately post-HPCT drastically diminish in the long term.[65] It has been suggested that donor subsets of NK cells may only express a single KIR in the initial 3 months following transplantation as part of the receptor acquisition process. Understanding the NK cell population's place in a post-transplant immune system is vital for understanding how this activity can be enhanced for improved post-transplant outcomes. Transplant centres are showing conflicting results when applying a standardised model of KIR assessment to their HPCT donor selection programmes, but this data is rarely published. Understanding the mechanisms of action could help us to understand why these conflicts in findings arise, and would aid the refinement of KIR assessment models so that they provide universal benefits to patients of all transplant centres.

While studies have focussed on the cytotoxic action of NK cells, these lymphocytes may also apply indirect effects via interactions with the adaptive immune system. A recent study into the control of viral load within infected cells by NK cells found that iKIR exerted influence upon the $CD8^+$ T cell response.[95] This study showed that HLA-B57 provides a protective quality, reducing the viral load in this patient cohort, and HLA-B35 presents a detrimental effect, with these individuals more susceptible to high viral loads. iKIR was found to exacerbate this effect, in both a beneficial capacity (in association with HLA-B57) and in a detrimental capacity (in association with HLA-B35), by extending the lifespan of $CD8^+$ T cells.[95] The iKIR on the surface of the T cell was found to have a direct effect on the T cell, but iKIR on NK cells may also exert an indirect influence.

It is not known whether this finding in viral immune defence has any implications for the transplantation setting. NK cells are the first lymphocyte to reconstitute in the recipient's immune system post-HPCT, considerably earlier than both T cells and B cells.[96] It is possible that NK cells could establish the post-transplant immunological environment for the adaptive immune system, potentially providing opportunity for therapeutic manipulation to enhance a GvL and/or anti-CMV effect. It is also possible that KIR on the surface of T cells may modulate their immune response for similar benefits. More research is required to investigate the interactions between NK cells and the wider immune system, especially in the context of transplantation.

5 | CONCLUSION

The study of KIR has progressed dramatically over the past 10 years, transforming our understanding of the role of NK cells in medical science, including the HPCT setting. NGS and other emerging technologies will now help the scientific community to further understand the basic science underlying the function of NK cells, and aid development of new clinical interventions to improve the therapeutic value of a wide range of medical services.

The role of NGS in routine clinical HPCT donor assessments is still unclear. It is possible that the broad gene presence/absence approach of current donor selection algorithms based on KIR gene content are the optimal balance between clinical efficacy, efficiency of testing, and fiscal pressures. The additional financial burden of NGS coupled with relatively long test turnaround times dictate that high-resolution results must provide a significant improvement in clinical outcomes to favour this technology over low resolution technologies with a rapid turnaround time, such as polymerase chain reaction-sequence specific oligonucleotides and real time PCR (qPCR). Nonetheless, the use of NGS analysis, cellular and molecular assays in combination offers an exciting opportunity for investigation into the interaction between KIR and HLA and how this relates to the proteomics and cellular function of NK cells. The current research into allelic variation within each KIR gene offers a glimpse into the deeper functions of KIR proteins, but further work is required to elucidate how this variation influences the functional capacity of different NK cell subpopulations.

Finally, despite great developments in the research of KIR, there remains a less than universal usage of a common nomenclature. Care must be taken to ensure published reference sets are used in published studies wherever possible to improve clarity when comparing studies. Further expansion of the work of Pyo et al[25] and Vierra-Green et al[24] to designate and apply a universal KIR haplotype nomenclature system would greatly aid this endeavour.

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CONFLICT OF INTEREST

The author has declared no conflicting interests.

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REFERENCES

1. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. 1986;319(6055):675-678.
2. Mandelboim O, Reyburn HT, Valés-Gómez M, et al. Protection from lysis by natural killer cells of group 1 and 2 specificity is mediated by residue 80 in human histocompatibility leukocyte antigen C alleles and also occurs with empty major histocompatibility complex molecules. *J Exp Med*. 1996;184:913-922.
3. Hilton HG, Guethlein LA, Goyos A, et al. Polymorphic HLA-C receptors balance the functional characteristics of KIR haplotypes. *J Immunol*. 2015;195(7):3160-3170.
4. Moesta AK, Norman PJ, Yawata M, Yawata N, Gleimer M, Parham P. Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *J Immunol*. 2008;180(6):3969-3979.
5. Hansasuta P, Dong T, Thananchai H, et al. Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. *Eur J Immunol*. 2004;34(6):1673-1679.
6. Saunders PM, Vivian JP, Baschuk N, et al. The interaction of KIR3DL1*001 with HLA class I molecules is dependent upon molecular microarchitecture within the Bw4 epitope. *J Immunol*. 2015;194(2):781-789.
7. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci U S A*. 1993;90(24):12000-12004.
8. Colonna M, Spies T, Strominger JL, et al. Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene. *Proc Natl Acad Sci U S A*. 1992;89(17):7983-7985.
9. Winter CC, Gumperz JE, Parham P, Long EO, Wagtmann N. Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J Immunol*. 1998;161:571-577.
10. Robinson J, Halliwell JA, Hayhurst JD, Flicek P, Parham P, Marsh SG. The IPD and IMGT/HLA database: allele variant databases. *Nucleic Acids Res*. 2015;43(Database issue):D423-D431.
11. Parham P, Norman PJ, Abi-Rached L, Guethlein LA. Human-specific evolution of killer cell immunoglobulin-like receptor recognition of major histocompatibility complex class I molecules. *Philos Trans R Soc Lond B Biol Sci*. 2012;367(1590):800-811.
12. Gendzekhadze K, Garcia-Gomez J, Cano P, Senitzer D. KIR shapes HLA. *Hum Immunol*. 2015;76:40.
13. Wroblewski EE, Parham P, Guethlein LA. Two to tango: co-evolution of hominid natural killer cell receptors and MHC. *Front Immunol*. 2019;10(177):1-22.
14. Martin MP, Gao X, Lee JH, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet*. 2002;31(4):429-434.
15. Stewart CA, Laugier-Anfossi F, Vély F, et al. Recognition of peptide-MHC class I complexes by killer immunoglobulin-like receptors. *Proc Natl Acad Sci U S A*. 2005;2005:13224-13229.
16. O'Connor GM, Vivian JP, Gostick E, et al. Peptide-dependent recognition of HLA-B*57:01 by KIR3DS1. *J Virol*. 2015;89(10):5213-5221.
17. Burian A, Wang KL, Finton KA, et al. HLA-F and MHC-I open conformers bind natural killer cell Ig-like receptor KIR3DS1. *PLoS One*. 2016;11(9):e0163297.
18. Dulberger CL, McMurtrey CP, Holzemer A, et al. Human leukocyte antigen F presents peptides and regulates immunity through interactions with NK cell receptors. *Immunity*. 2017;46(6):1018-1029 e1017.
19. Thiruchelvam-Kyle L, Hoelsbrekken SE, Saether PC, et al. The activating human NK cell receptor KIR2DS2 recognizes a beta2-microglobulin-independent ligand on cancer cells. *J Immunol*. 2017;198(7):2556-2567.
20. Gonzalez-Galarza FF, Takeshita LY, Santos EJ, et al. Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. *Nucleic Acids Res*. 2015;43(Database issue):D784-D788.
21. Gomez-Lozano N, Estefania E, Williams F, et al. The silent KIR3DP1 gene (CD158c) is transcribed and might encode a secreted receptor in a minority of humans, in whom the KIR3DP1, KIR2DL4 and KIR3DL1/KIR3DS1 genes are duplicated. *Eur J Immunol*. 2005;35(1):16-24.
22. Marsh SG, Parham P, Dupont B, et al. Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Immunogenetics*. 2003;55(4):220-226.
23. Vierra-Green C, Roe D, Jayaraman J, et al. Estimating KIR haplotype frequencies on a cohort of 10,000 individuals: a comprehensive study on population variations, typing resolutions, and reference haplotypes. *PLoS One*. 2016;11(10):e0163973.
24. Vierra-Green C, Roe D, Hou L, et al. Allele-level haplotype frequencies and pairwise linkage disequilibrium for 14 KIR loci in 506 European-American individuals. *PLoS One*. 2012;7(11):e47491.
25. Pyo CW, Guethlein LA, Vu Q, et al. Different patterns of evolution in the centromeric and telomeric regions of group A and B haplotypes of the human killer cell Ig-like receptor locus. *PLoS One*. 2010;5(12):e15115.

26. Sambrook JG, Bashirova A, Andersen H, et al. Identification of the ancestral killer immunoglobulin-like receptor gene in primates. *BMC Genomics*. 2006;7:209.
27. Uhrberg M, Valiante NM, Shum BP, et al. Human diversity in killer cell inhibitory receptor genes. *Immunity*. 1997;7:753-763.
28. Cichocki F, Miller JS, Anderson SK. Killer immunoglobulin-like receptor transcriptional regulation: a fascinating dance of multiple promoters. *J Innate Immun*. 2011;3(3):242-248.
29. Manser AR, Weinhold S, Uhrberg M. Human KIR repertoires: shaped by genetic diversity and evolution. *Immunol Rev*. 2015; 267(1):178-196.
30. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol*. 2002;20: 217-251.
31. Vilches C, Pando MJ, Parham P. Genes encoding human killer-cell Ig-like receptors with D1 and D2 extracellular domains all contain untranslated pseudoexons encoding a third Ig-like domain. *Immunogenetics*. 2000;51(8–9):639-646.
32. Vilches C, Gardiner CM, Parham P. Gene structure and promoter variation of expressed and nonexpressed variants of the KIR2DL5 gene. *J Immunol*. 2000;165(11):6416-6421.
33. Selvakumar A, Steffens U, Dupont B. NK cell receptor gene of the KIR family with two IG domains but highest homology to KIR receptors with three IG domains. *Tissue Antigens*. 1996;48 (4 Pt 1):285-294.
34. Trundley AE, Hiby SE, Chang C, et al. Molecular characterization of KIR3DL3. *Immunogenetics*. 2006;57(12):904-916.
35. Wilson MJ, Torkar M, Haude A, et al. Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci U S A*. 2000;97(9):4778-4783.
36. Hsu KC, Liu XR, Selvakumar A, Mickelson E, O'Reilly RJ, Dupont B. Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J Immunol*. 2002;169(9):5118-5129.
37. Jiang W, Johnson C, Jayaraman J, et al. Copy number variation leads to considerable diversity for B but not A haplotypes of the human KIR genes encoding NK cell receptors. *Genome Res*. 2012;22(10):1845-1854.
38. Hsu KC, Chida S, Geraghty DE, Dupont B. The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev*. 2002;190:40-52.
39. EMBL-EBI. Statistics for release 2.8.0 (December 2019) 2018.
40. Hilton HG, Norman PJ, Nemat-Gorgani N, et al. Loss and gain of natural killer cell receptor function in an African hunter-gatherer population. *PLoS Genet*. 2015;11(8):e1005439.
41. Misra MK, Augusto DG, Martin GM, et al. Report from the killer-cell immunoglobulin-like receptors (KIR) component of the 17th International HLA and Immunogenetics Workshop. *Hum Immunol*. 2018;79(12):825-833.
42. Maxwell LD, Wallace A, Middleton D, Curran MD. A common KIR2DS4 deletion variant in the human that predicts a soluble KIR molecule analogous to the KIR1D molecule observed in the rhesus monkey. *Tissue Antigens*. 2002;60(3):254-258.
43. Middleton D, Gonzalez A, Gilmore PM. Studies on the expression of the deleted KIR2DS4*003 gene product and distribution of KIR2DS4 deleted and nondeleted versions in different populations. *Hum Immunol*. 2007;68(2):128-134.
44. Traherne JA, Martin M, Ward R, et al. Mechanisms of copy number variation and hybrid gene formation in the KIR immune gene complex. *Hum Mol Genet*. 2010;19(5):737-751.
45. Béziat V, Traherne JA, Liu LL, et al. Influence of KIR gene copy number on natural killer cell education. *Blood*. 2013;121 (23):4703-4707.
46. Gamazon ER, Stranger BE. The impact of human copy number variation on gene expression. *Brief Funct Genomics*. 2015;14(5): 352-357.
47. Vendelbosch S, de Boer M, Gouw RA, et al. Extensive variation in gene copy number at the killer immunoglobulin-like receptor locus in humans. *PLoS One*. 2013;8(6):e67619.
48. Santourlidis S, Trompeter HI, Weinhold S, et al. Crucial role of DNA methylation in determination of clonally distributed killer cell Ig-like receptor expression patterns in NK cells. *J Immunol*. 2002;169(8):4253-4261.
49. Chan HW, Kurago ZB, Stewart CA, et al. DNA methylation maintains allele-specific KIR gene expression in human natural killer cells. *J Exp Med*. 2003;197(2):245-255.
50. Davies GE, Locke SM, Wright PW, et al. Identification of bidirectional promoters in the human KIR genes. *Genes Immun*. 2007;8(3):245-253.
51. Trompeter HI, Gomez-Lozano N, Santourlidis S, et al. Three structurally and functionally divergent kinds of promoters regulate expression of clonally distributed killer cell Ig-like receptors (KIR), of KIR2DL4, and of KIR3DL3. *J Immunol*. 2005;174 (7):4135-4143.
52. Horowitz A, Strauss-Albee DM, Leipold M, et al. Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. *Sci Transl Med*. 2013;5(208):208ra145.
53. Howard CA, Fernandez-Vina MA, Appelbaum FR, et al. Recommendations for donor human leukocyte antigen assessment and matching for allogeneic stem cell transplantation: consensus opinion of the Blood and Marrow Transplant Clinical Trials Network (BMT CTN). *Biol Blood Marrow Transplant*. 2015;21 (1):4-7.
54. Mayor NP, Hayhurst JD, Turner TR, et al. Recipients receiving better HLA-matched hematopoietic cell transplantation grafts, uncovered by a novel HLA typing method, have superior survival: a retrospective study. *Biol Blood Marrow Transplant*. 2019;25(3):443-450.
55. Shaw BE, Mayor NP, Szydlo RM, et al. Recipient/donor HLA and CMV matching in recipients of T-cell-depleted unrelated donor haematopoietic cell transplants. *Bone Marrow Transplant*. 2017;52(5):717-725.
56. Cooley S, Trachtenberg E, Bergemann TL, et al. Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood*. 2009;113(3):726-732.
57. Symons HJ, Leffell MS, Rossiter ND, Zahurak M, Jones RJ, Fuchs EJ. Improved survival with inhibitory killer immunoglobulin receptor (KIR) gene mismatches and KIR haplotype B donors after nonmyeloablative, HLA-haploidentical bone marrow transplantation. *Biol Blood Marrow Transplant*. 2010;16(4): 533-542.
58. Oevermann L, Michaelis SU, Mezger M, et al. KIR B haplotype donors confer a reduced risk for relapse after haploidentical transplantation in children with ALL. *Blood*. 2014;124(17): 2744-2747.

59. Cooley S, Weisdorf DJ, Guethlein LA, et al. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood*. 2010;116(14):2411-2419.
60. Cooley S, Weisdorf DJ, Guethlein LA, et al. Donor killer cell Ig-like receptor B haplotypes, recipient HLA-C1, and HLA-C mismatch enhance the clinical benefit of unrelated transplantation for acute myelogenous leukemia. *J Immunol*. 2014;192(10):4592-4600.
61. Zhou H, Bao X, Wu X, et al. Donor selection for KIR B haplotype of the centromeric motifs can improve the outcome after HLA-identical sibling hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2014;20:98-105.
62. Bao X, Wang M, Zhou H, Zhang H, Wu X, He J. Donor KIR genotype Bx1 had a negative effect while centromeric B-specific gene motifs had a positive effect of survival for standard risk AML patients after unrelated donor hemotopoietic stem cell transplantation. *Hum Immunol*. 2015;76:75.
63. Weisdorf D, Cooley S, Wang T, et al. KIR donor selection: feasibility in identifying better donors. *Biol Blood Marrow Transplant*. 2019;25(1):e28-e32.
64. EMBL-EBI. Donor KIR B-content group calculator 2017.
65. Leung W, Iyengar R, Turner V, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol*. 2004;172(1):644-650.
66. Park S, Kim K, Jang JH, et al. KIR alloreactivity based on the receptor-ligand model is associated with improved clinical outcomes of allogeneic hematopoietic stem cell transplantation: result of single center prospective study. *Hum Immunol*. 2015;76(9):636-643.
67. Faridi RM, Kemp TJ, Dharmani-Khan P, et al. Donor-recipient matching for KIR genotypes reduces chronic GVHD and missing inhibitory KIR ligands protect against relapse after myeloablative, HLA matched hematopoietic cell transplantation. *PLoS One*. 2016;11(6):e0158242.
68. Gianchecchi E, Delfino DV, Fierabracci A. NK cells in autoimmune diseases: linking innate and adaptive immune responses. *Autoimmun Rev*. 2018;17(2):142-154.
69. Leung W, Handgretinger R, Iyengar R, Turner V, Holladay MS, Hale GA. Inhibitory KIR-HLA receptor-ligand mismatch in autologous haematopoietic stem cell transplantation for solid tumour and lymphoma. *Br J Cancer*. 2007;97(4):539-542.
70. Ruggeri L, Capanni M, Casucci M, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood*. 1999;94(1):333-339.
71. Wu S, Zeng YJ, Zhang C, Deng TX, Xu YQ, Zhang X. The role of the killer cell immunoglobulin-like receptor (KIR) "missing self" model in unrelated donor HSCT: a meta-analysis. *Transplant Proc*. 2015;47(2):558-565.
72. Yahng SA, Jeon YW, Yoon JH, et al. Negative impact of unidirectional host-versus-graft killer cell immunoglobulin-like receptor ligand mismatch on transplantation outcomes after unmanipulated haploidentical peripheral blood stem cell transplantation for acute myeloid leukemia. *Biol Blood Marrow Transplant*. 2016;22(2):316-323.
73. Nowak J, Koscinska K, Mika-Witkowska R, et al. Donor NK cell licensing in control of malignancy in hematopoietic stem cell transplant recipients. *Am J Hematol*. 2014;89(10):E176-E183.
74. Nowak J, Koscinska K, Mika-Witkowska R, et al. Role of donor activating KIR-HLA ligand-mediated NK cell education status in control of malignancy in hematopoietic cell transplant recipients. *Biol Blood Marrow Transplant*. 2015;21(5):829-839.
75. Tu MM, Mahmoud AB, Makrigiannis AP. Licensed and unlicensed NK Cells: differential roles in cancer and viral control. *Front Immunol*. 2016;7:166.
76. Boudreau JE, Hsu KC. Natural killer cell education and the response to infection and cancer therapy: stay tuned. *Trends Immunol*. 2018;39(3):222-239.
77. Sahin U, Dalva K, Gungor F, Ustun C, Beksac M. Donor-recipient killer immunoglobulin like receptor (KIR) genotype matching has a protective effect on chronic graft versus host disease and relapse incidence following HLA-identical sibling hematopoietic stem cell transplantation. *Ann Hematol*. 2018;97(6):1027-1039.
78. Sobecks RM, Wang T, Askar M, et al. Impact of KIR and HLA genotypes on outcomes after reduced-intensity conditioning hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2015;21(9):1589-1596.
79. Neuchel C, Furst D, Niederwieser D, et al. Impact of donor activating KIR genes on HSCT outcome in C1-ligand negative myeloid disease patients transplanted with unrelated donors - a retrospective study. *PLoS One*. 2017;12(1):e0169512.
80. Arima N, Kanda J, Tanaka J, et al. Homozygous HLA-C1 is associated with reduced risk of relapse after HLA-matched transplantation in patients with myeloid leukemia. *Biol Blood Marrow Transplant*. 2018;24(4):717-725.
81. Park H, Rho EY, In JW, et al. The impact of HLA and KIR ligand mismatching on unrelated allogeneic hematopoietic stem cell transplantation in Korean adult patients. *Ann Lab Med*. 2015;35(1):111-117.
82. Fischer JC, Ottinger H, Ferencik S, et al. Relevance of C1 and C2 epitopes for hemopoietic stem cell transplantation: role for sequential acquisition of HLA-C-specific inhibitory killer Ig-like receptor. *J Immunol*. 2007;178(6):3918-3923.
83. Miller JS, McCullar V. Human natural killer cells with polyclonal lectin and immunoglobulin like receptors develop from single hematopoietic stem cells with preferential expression of NKG2A and KIR2DL2/L3/S2. *Blood*. 2001;98(3):705-713.
84. Arima N, Nakamura F, Yabe T, et al. Influence of differently licensed KIR2DL1-positive natural killer cells in transplant recipients with acute leukemia: a Japanese national registry study. *Biol Blood Marrow Transplant*. 2016;22(3):423-431.
85. Boudreau JE, Mulrooney TJ, Le Luduec JB, Barker E, Hsu KC. KIR3DL1 and HLA-B density and binding calibrate NK education and response to HIV. *J Immunol*. 2016;196(8):3398-3410.
86. Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P. The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. *J Immunol*. 2003;171(12):6640-6649.
87. Wagner I, Schefzyk D, Pruschke J, et al. Allele-level KIR genotyping of more than a million samples: workflow, algorithm, and observations. *Front Immunol*. 2018;9(2843):1-15.

88. Alicata C, Pende D, Meazza R, et al. Hematopoietic stem cell transplantation: improving alloreactive Bw4 donor selection by genotyping codon 86 of KIR3DL1/S1. *Eur J Immunol*. 2016;46(6):1511-1517.
89. Taner SB, Pando MJ, Roberts A, et al. Interactions of NK cell receptor KIR3DL1*004 with chaperones and conformation-specific antibody reveal a functional folded state as well as predominant intracellular retention. *J Immunol*. 2011;186(1):62-72.
90. Wu X, Yao Y, Bao X, et al. KIR2DS4 and its variant KIR1D are associated with acute graft-versus-host disease, cytomegalovirus, and overall survival after sibling-related HLA-matched transplantation in patients with donors with KIR gene haplotype A. *Biol Blood Marrow Transplant*. 2016;22(2):220-225.
91. Burek Kamenaric M, Stingl Jankovic K, Grubic Z, et al. The impact of KIR2DS4 gene on clinical outcome after hematopoietic stem cell transplantation. *Hum Immunol*. 2017;78(2):95-102.
92. Bari R, Rujkijyanont P, Sullivan E, et al. Effect of donor KIR2DL1 allelic polymorphism on the outcome of pediatric allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol*. 2013;31(30):3782-3790.
93. Gumperz JE. Conserved and variable residues within the Bw4 motif of HLA-B make separable contributions to recognise by the NKB1 kill cell-inhibitory receptor. *J Immunol*. 1997;158:5237-5241.
94. Sanjanwala B, Draghi M, Norman PJ, Guethlein LA, Parham P. Polymorphic sites away from the Bw4 epitope that affect interaction of Bw4+ HLA-B with KIR3DL1. *J Immunol*. 2008;181(9):6293-6300.
95. Boelen L, Debebe B, Silveira M, et al. Inhibitory killer cell immunoglobulin-like receptors strengthen CD8(+) T cell-mediated control of HIV-1, HCV, and HTLV-1. *Sci Immunol*. 2018;3(29):eaao2892.
96. Nguyen S, Dhedin N, Vernant JP, et al. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. *Blood*. 2005;105(10):4135-4142.

AUTHOR BIOGRAPHY

Paul A. Wright is a senior clinical scientist in Histocompatibility & Immunogenetics at the Transplantation Laboratory, Manchester University NHS Foundation Trust, United Kingdom. He received a BSc in Microbiology from the University of Manchester and a MSc in Transfusion & Transplantation Sciences from the University of Bristol, before commencing post-graduate clinical scientist training in 2010. He registered as a clinical scientist in 2014, and attained diplomate status with the Royal College of Pathologists in 2018. During his 10 years working in clinical H&I, Mr Wright has supported local services in both solid organ and haematopoietic progenitor cell transplantation (HPCT). In 2014, Mr Wright enrolled in the NHS Higher Specialist Scientist Training programme, where he focused on the influence of the immunogenetics of the natural killer cells' KIR gene system on HPCT as part of a professional doctorate thesis in association with the University of Manchester. He currently supervises the next generation sequencing services of the Manchester Transplantation Laboratory.

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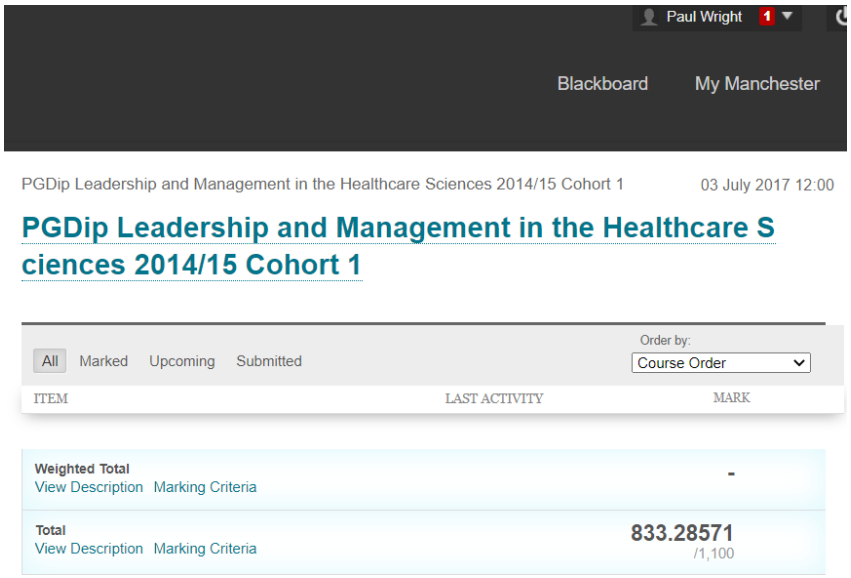
Appendix E – Evidence of Remaining Doctorate in Clinical Science Components

Appendix E.i – Module A Results Overview

Alliance Manchester Business School (AMBS) **A Units**		
Unit Title	**Credits**	**Assignment Word Count**
A1: Professionalism and Professional Development in the Healthcare Environment	30	Practice Paper – 2000 words A1 – Assignment 1 – 1500 words A1 – Assignment 2 – 4000 words
A2: Theoretical Foundations of Leadership	20	A2 – Assignment 1 – 3000 words A2 – Assignment 2 – 3000 words
A3: Personal and Professional Development to Enhance Performance	30	A3 – Assignment 1 – 1500 words A3 – Assignment 2 – 4000 words
A4: Leadership and Quality Improvement in the Clinical and Scientific Environment	20	A4 – Assignment 1 – 3000 words A4 – Assignment 2 – 3000 words
A5: Research and Innovation in Health and Social care	20	A5 – Assignment 1 – 3000 words A5 – Assignment 2 – 3000 words

Life Sciences – Section C

C1: Innovation Project – Credits = 70 Assignment: Literature Review/ & Lay Presentation



Appendix E.ii – HSST Module B: Royal College Part I Examination Certificate

The Royal College of Pathologists

By these letters make it known that

Paul Wright

having undertaken the required training and
after having passed the Part One examination in

Histocompatibility & Immunogenetics

has been awarded

Associateship of
The Royal College of Pathologists

In witness whereof the Seal of the College and the signatures
of the proper Officers have been affixed this first day of February 2018

LONDON

President *Registrar* *Member of Council*

Business case for high resolution typing of KIR genes as part of the haematopoietic progenitor cell transplantation service provided by the Transplantation Laboratory

Paul Andrew Wright

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Submitted: 30/06/2017

Submitted as part of University of Manchester/MAHSE/NHS HSST Scheme

I. Opportunity

Only approximately 1 in 3 patients awaiting Haematopoietic Progenitor Cell Transplantation (HPCT) have access to a fully HLA matched sibling – the ideal choice of donor. The remaining patients will require a transplant from either a non-HLA matched relative or a HLA-Matched Unrelated Donor (MUD). In these cases, donor selection algorithms are vital to ensure the patient has the highest likelihood of a successful transplant outcome.

Matching the HLA genes of recipient and potential donor to an allelic level to universally agreed to be the primary criterion for HPC donor selection, but there remains capacity to further improve selection algorithms to ensure all patients receive transplants from the optimal available donors, as even patients receiving 10/10 HLA matched transplants risk experiencing complications such as relapse of malignancy or graft versus host disease.

Research into the impact of the KIR gene region on HPCT has grown significantly over the past decade. KIR genes can be categorised as exhibiting either an inhibitory or activating effect on Natural Killer (NK) cell activation. KIR profiles will vary between individuals, by both gene content and allelic polymorphisms, resulting in diversity which can be used to select donors. In the broadest sense, individuals can be stratified by whether they possess A and/or B haplotypes. A haplotypes possess only one activating KIR gene, and B haplotypes possess several activating KIR genes. Promoting NK cell activation, by selecting donors with more activating KIR genes, is known to improve post-transplant outcomes, and so B haplotypes are preferred. This has been developed into the B haplotype Scoring System, which categorises the KIR genes of potential donors as "neutral", "better", or "best" on the basis of whether the donor possesses A and/or B haplotypes. This system has been used locally since 2015.

Recent studies have suggested polymorphisms within KIR genes offer further data to inform HPCT donor selection algorithms. Our department's current method for genotyping KIR genes can only identify the presence or absence of each KIR gene (the gene content), and cannot define the alleles each individual possesses. By attaining high resolution genotyping of KIR genes, we will be able to implement these new findings into our services and develop cutting-edge research within the Trust to ensure we meet the Central Manchester NHS Foundation Trust's vision of leading healthcare, excelling in quality, research, and innovation.

Currently no laboratory within the NHS offers high resolution KIR genotyping services. It is known that several associated organisations, such as Anthony Nolan and the DKMS (German bone marrow registry) are in the process of developing and implementing this technology. It is essential that the NHS do not fall behind in meeting modern expectations for providing cutting-edge healthcare.

The Manchester Transplantation Laboratory has recently purchased equipment for Next Generation Sequencing (NGS) of HLA genes using the Illumina MiSeq platform. This equipment could also be used for NGS of the KIR region, providing high resolution genotyping of KIR genes without the expense of purchasing additional equipment. The Illumina MiSeq is therefore considered the most cost effective solution for high resolution genotyping of KIR genes within our department. The purchase of new equipment solely for the typing of KIR would not be a justifiable expense in the context of the resources currently available within the department.

II. Objectives and Metrics

The primary objective is to purchase technology which can provide high resolution allelic genotyping of the KIR gene region. Within this aim, a number of metrics must be considered before purchasing can be finalised:

1. Cost Effectiveness – Is the technology financially viable as a service
2. Workflow – What is the turnaround for testing and reporting results? Does the workflow function within the current laboratory structure?
3. Labour Intensity – How much staff time is required for testing? How experienced/skilled are the staff required to be? Does the labour requirement fit within the current laboratory staffing structure or are additional trained personnel required to provide a satisfactory service?
4. Repeat Rates – Repeat rates reduce the cost effectiveness of the test, increase the turnaround time for testing, and potentially waste staff time. Maintaining minimal repeat rates is essential for providing an efficient service.
5. High Sensitivity and Specificity – A high sensitivity ensures high resolution genotyping is achieved, and high specificity ensures the test is reliable.

III. Stakeholder Engagement

We currently provide a KIR genotyping service to our service users, providing results on donor/recipient KIR compatibility based upon the KIR B Haplotype Scoring Model. All interpretation of KIR haplotypes is carried out within the laboratory, with this interpretation communicated to clinicians via a report. Upon informal discussion with the clinicians of the children's and adult haematology service, it was agreed that any development in technologies would be welcomed on the basis that future developments to the service provided the same or improved data to aid patient services, and this improvement was cost effective.

The Transplantation Laboratory currently provides KIR compatibility results for 71 samples in 2016. This activity is likely to rise in future years due to the growing popularity of haploidentical HPCT.

IV. Potential Solutions

Upon assessing our current practice and available technologies, potential options to consider are as follows:

A. Maintain current practice – Luminex PCR-RSSO genotyping Continue using Luminex RSSO technology to identify presence or absence of KIR genes without defining to the allelic level.
B. Real time PCR (qPCR) qPCR kits are available from Linkage Bioscience. This technique is also only for identification of the presence/absence of KIR genes without defining to the allelic level, but may be considered due to ease of use.
C. Commercial kit for NGS of KIR region compatible with Illumina MiSeq platform Though there is no commercial NGS kit for the KIR gene region using the Illumina MiSeq platform currently available, both Stanford University and Illumina are currently developing a NGS kit to bring to market.
D. Establish an in-house kit for NGS of KIR region Though this would be slower, more labour intensive than purchasing a commercial kit, an in-house approach would potentially offer significant cost savings in comparison to commercial kits. Developing an in-house kit would involve the selection of primers, and developing a sequencing protocol that ensures high sensitivity and specificity with minimal background interference, followed by validation of the test for clinical use.

V. Initial Assessment of Solutions

The current practice of using PCR-RSSO to identify the absence or presence of KIR genes (Option A) provides limited scope for HPC donor assessment. Achieving high resolution genetic typing would future-proof our donor selection processes, and would allow us to carry out cutting-edge research to further the field of transplantation. It is of note that this technique has failed to correctly identify the KIR profile of two External Quality Assessment samples over the past 5 years, bringing the sensitivity and safety of the test into question. As such, maintaining current practice should not be considered sufficient to guarantee the future quality of the service.

Similarly, the use of qPCR (Option B) does not provide any functional advantage in results provided compared to PCR-RSSO. This technique is currently being considered as a potential interim methodology until the use of high resolution typing has been validated and put into routine use. Utility as an interim measure will only be considered if qPCR provides significant advantages over PCR-RSSO.

Upon contacting Stanford University, Illumina, and GenDX for information concerning their commercial NGS kits, no organisation was in a position to offer sufficient information for considering this option (Option C) at this time. Stanford University and GenDX declined to provide a date for release, while Illumina stated release would be "at least 12 months". As such, this option is not viable at this time. If NGS of the KIR region is to be utilised within our department in the foreseeable future, we must develop an in-house kit (Option D). Developing an in-house kit would take time and would be highly labour intensive, but once established, would provide significant cost savings.

The cost of developing an in-house NGS kit would require consideration of labour cost dedicated to the development process. However, this project would be suitable as part of a Higher Specialist Scientific Training (HSST) trainee's doctorate – of which the department is supportive of providing time for training. As such, labour costs of development can be ignored in this case.

VI. In-Depth Assessment of Solutions

VIa. Financial Assessment

Full calculations of costings can be found in appendix i. Labour costs are not considered in these pricings.

Technology	**Cost per sample**
Current Technology: Luminex LABType® PCR-RSSO KIR Genotyping (One Lambda Inc) (Presence and Absence of KIR genes)	Approx. £48 (Range £48-55)
Prospective Technology: Illumina LinkSeq® qPCR KIR Genotyping (Linkage Biosystems™) (Presence and Absence of KIR genes)	Two tests per tray: £32.85 *(If discounted: £29.57*)* One test per tray: £37.08 *(If discounted £33.37*)* **In the past, Linkage Biosystems have provided ~10% discount on their products*
Prospective Technology: NGS KIR genotyping (Illumina®) (Sequencing of KIR alleles to low resolution)	£60 (initial pricing from illumina)

The cost of PCR-RSSO KIR Genotyping is based upon current routine procedures used within the department, considering the price of testing control samples and of repeating failed tests.

LinkSeq® qPCR trays are available as one or two kits per tray. The two test trays are more cost effective per sample, but both variants would be required to allow flexibility for laboratory testing (e.g. if an odd number of samples were to be tested). Therefore, the true cost per sample considering the use of both variants is like to be somewhere between the two values, though the variance in price is minor.

NGS genotyping is the most expensive technology, but also provides more KIR data. As such, the additional expense would be considered worthwhile for the additional research prospects and potential for expanding services (e.g. KIR allele disease associations) and future-proofing our services so that we can rapidly respond to relevant research findings.

VIb. Workflow, Labour Intensity, and Labour Costs Assessment

Gantt charts were carried out as part of the assessment of these techniques (Appendix ii). The HLA NGS method was used as a model for timing of KIR NGS.

The consideration of labour costs for carrying out PCR-RSSO KIR genotyping is negligible, as these samples are carried out within a multi-batch, in parallel with PCR-RSSO HLA genotyping. However, interpretation of data is relatively slow due to the test's propensity for false positive and false negative results. Repeat rates are also relatively high at 14%.

The LinkSeq® qPCR is a relatively rapid test, with approx. 15-20minute set up time (carried out by a band 4 or 5 staff, and observed by a band 7 staff if multiple samples are tested), and automated analysis within 5-10 minutes (Carried out by a band 7 staff and checked by band 8 staff). Due to the rapid set up time, this work can be easily allocated within our current staffing strategy with minimum stress upon services.

Though the turn-around time of KIR NGS is long, it is of note that KIR and HLA tests can be compiled onto a single sequencing reaction micro-plate following compilation of libraries. This would minimise the cost of labour required per sample, and streamline the use of equipment. Further analysis is required to assess how compiling KIR and HLA NGS testing would impact upon use of equipment.

VIc. Initial Trial of LinkSeq® qPCR kits

Six sample LinkSeq tests were provided by Linkage Biosystems® for an initial trial assessment. This assessment was highly positive, with the speed of set up and analysis noted as improvements over the PCR-RSSO kits (report available upon request). Six External Quality Assessment (EQA) samples were tested, including one sample that the department scored an unsatisfactory result with PCR-RSSO technique (due to two false positive KIR gene results).

All six tests were concordant with the consensus result with no manual manipulations. Further validation testing will be required to rigorously assess repeat rates and concordance with known results, but initial results suggest the LinkSeq® qPCR technology may be faster and more reliable than PCR-RSSO. The primary disadvantage of qPCR technology is the requirement for a dedicated member of staff to set up these tests, but this is a minor issue due to the short set-up time.

VII. Proposed Action

Due to the improved turnaround time and improved sensitivity of LinkSeq® qPCR (Linkage Biosystems®) in comparison with Luminex LABType® PCR-RSSO (One Lambda Inc.), we propose that LinkSeq® qPCR should be considered as a replacement to the current testing method in the short term. This would require ordering additional reagents for validation, but could be completed within 3 months of receipt. This should be carried out swiftly due to our previous experience of non-conformance with EQA schemes on two samples using PCR-RSSO, and therefore has safety implications for our services.

In the medium to long term, an in-house NGS kit should be developed to provide high resolution allele data of our HPCT patients and donors. This would place us at the fore-front of medical research, helping us meet the vision of the Central Manchester NHS Foundation Trust to "be recognised internationally as leading healthcare; excelling in quality, safety, patient experience, research, innovation and teaching; dedicated to improving health and well-being for our diverse population.". The research this would facilitate would improve the services we can provide to our patients, reducing the need to implement costly interventional treatment for post-transplant patients due to complications such as relapse and graft versus host disease. No private industry is currently providing a commercial NGS kit for KIR genotyping, and so an in-house kit could make us a world-leader in this field.

Developing an NGS kit in-house could also potentially provide future revenues for the NHS, in the form of a potential commercial kit that could be released independently or in collaboration with an industrial partner organisation.

IV. Executive Summary

In stem cell transplantation, it is well understood that compatibility between the recipient and donor immune systems is essential for providing the optimal quality transplant for the recipient. HLA genes are well characterised as the primary compatibility indicator in transplantation, but over the past ten years there is growing evidence that KIR genes may provide a secondary compatibility indicator to further improve results. These improvements to compatibility significantly decrease the likelihood of patients developing severe post-transplant complications, such as relapse of cancer or graft versus host disease (an extremely painful and debilitating reaction of the skin, gastrointestinal tract, and liver).

Currently, the Transplantation Laboratory at Manchester is able to identify the KIR gene content (the KIR genes that are present) of the recipient and donor. However, there are a variety of alleles (forms) of each KIR gene – data that cannot be defined by the currently in use technology. New technology now makes it possible to sequence the KIR genes to identify the variant alleles to provide more information for research and compatibility of recipients and donors. This technology is not currently in use for transplantation within the NHS, making Manchester a major innovator in this practice.

This improvement does not only have clear implications for improving patient care, but also has cost benefits for the NHS. When a patient experiences severe complications post-transplant, they not only must be hospitalised, but also require costly intervention therapies in the form of medication, donor-lymphocyte infusions, and in some cases, a re-transplant. If the need for these interventions could be minimised by improving donor selection, there would be great cost savings for the haematology services. Prevention is better than treatment. Please aid us in improving patient care, and help the CMFT become a world-leader in KIR clinical research.

Appendices

ia. Calculations for cost of KIR PCR-RSSO genotyping per sample

Unit Price	£1,157
Unit microbead volume	160ul
Microbead volume used per tes	2ul
Pipette Loss per unit (approx)	5ul
Total bead volume accounting for pipette loss	155
Number of tests per unit	77.5
Number of tests rounded down to complete tests	77
Average run = 2.5 samples + 1 control	
so 1 run = 3.5 samples approx	
Number of runs per unit	22
Number of runs, accounting for duplicates	11
Cost per run	£105.18
Cost per sample	£42.07
Repeat rate = 10 out of 71 in last year (db figures)	14%
Cost per sample accounting for repeats	**£47.96**

Range of cost	
2 samples per run	
Number of runs per unit	25.66667
Number of runs, accounting for duplicates	12.83333
Number of runs, rounding down to complete runs	12
Cost per run (1 sample one control)	£96.42
Cost per sample	£48.21
Cost per sample accounting for repeats	**£54.96**
3 samples per run	
Total number of runs	19.25
Number of runs, accounting for duplicates	9.625
Number of runs, rounding down to complete runs	9
Cost per run (3 sample one control)	£128.56
Cost per sample	£42.85
Cost per sample accounting for repeats	**£48.85**

ib. **Calculations for cost of KIR LinkSeq® qPCR genotyping per sample**

Price per Test	**£32.85**
THIS IS LIST PRICE, NOT QUOTE	
If 10% discount	**£29.57**

1 test per plate	
Unit Price	$480
Tests per Unit	10
Price per Test	$48
Price per Test	**£37.08**
THIS IS LIST PRICE, NOT QUOTE	
If 10% discount	**£33.37**

ii Gantt chart for PCR-RSSO, LinkSeq® qPCR and NGS

Key

Technologist (band 4 or 5)
Clinical Scientist (b7)
Senior C.S. (b8)
Tech. (observed by CS)
Automated

LinkSeq qPCR

	Minutes											
Task	10	20	30	40	50	60	70	80	90	100	110	120
PCR set up												
PCR and Melt Curve												
Analysis												
Checking												

LABType PCR-RSSO

	Minutes																								
Task	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260
PCR set up																										
PCR																										
Batch data uploading onto system																										
Microbead binding steps																										
Luminex reading tests																										
Analysis																										
Checking																										

NGS

Day 1 – DNA Quantification

	Hour															
Task	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8
Setting up PCR																
PCR Reaction																11hr automated (overnight)

Day 2 – Cleaning Samples

	Hour															
Task	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8
Clean up PCR amplicons			(Short period observed by clinical scientist at end ~10min)													
Normalising amplicons																
Tagment amplicons					(Short period observed by clinical scientist at beginning and end ~15min total)											
Pooling amplicons and clean up							(Short period observed by clinical scientist at beginning ~10min total)									
Amplification PCR																
Clean up PCR																
Library Pooling																
Sequencing																~24hr automated (overnight)